Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 5687–5690, June 1993
Immunology



Appendix A #14

# Constitutive expression of B7 restores immunogenicity of tumor cells expressing truncated major histocompatibility complex class II molecules

(CD28/tumor immunity)

SIVASUBRAMANIAN BASKAR*, SUZANNE OSTRAND-ROSENBERG*†, NASRIN NABAVI‡, LEE M. NADLER§, GORDON J. FREEMAN§, AND LAURIE H. GLIMCHER¶

*Department of Biological Sciences, University of Maryland, Baltimore, MD 21228; ‡Department of Immunopharmacology, Roche Research Center, Nutley, NJ 07110-1199; §Division of Tumor Immunology, Dana–Farber Cancer Institute, Boston, MA 02115; and ¶Department of Cancer Biology, Harvard School of Public Health, and Department of Medicine, Harvard Medical School, Boston, MA 02115

*Communicated by Leroy Hood, March 12, 1993*

**ABSTRACT** **The inability of the autologous host to reject resident tumor cells is frequently the result of inadequate generation of tumor-specific T cells. Specific activation of T cells occurs after delivery of two signals by the antigen-presenting cell. The first signal is antigen-specific and is the engagement of the T-cell antigen receptor by a specific major histocompatibility complex antigen–peptide complex. For some T cells, the second or costimulatory signal is the interaction of the T-cell CD28 receptor with the B7 activation molecule of the antigen-presenting cell. In the present study, we demonstrate that mouse sarcoma cells genetically engineered to provide both T-cell activation signals stimulate potent tumor-specific CD4+ T cells that cause rejection of both engineered and wild-type neoplastic cells. Two other recent studies have also demonstrated that costimulation via B7 can improve tumor immunity. However, our study differs from these reports by two important observations. (*i*) One of these studies utilized mouse tumor cells expressing xenogeneic viral antigens, and hence, the results are not applicable to wild-type resident tumors. Our study, however, demonstrates that coexpression of B7 by major histocompatibility complex class II+ tumor cells induces immunity in the autologous host that is specific for naturally occurring tumor antigens of poorly immunogenic tumors. (*ii*) In both earlier studies, only CD8+ T cells were activated after coexpression of B7, whereas in the present report, tumor-specific CD4+ T cells are generated. This report therefore illustrates the role of the B7 activation molecule in stimulating potent tumor-specific CD4+ T cells that mediate rejection of wild-type tumors and provides a theoretical basis for immunotherapy of established tumors.**

Rejection of a tumor by the autologous host is often mediated by tumor-specific T lymphocytes. Recent studies from a number of laboratories (1–3) suggest that the inability of the host to reject a resident tumor may be due to the insufficient generation of tumor-specific T helper lymphocytes. CD4+ T helper cells are specifically activated when they receive two signals delivered by an appropriate antigen-presenting cell (APC) (4). The first signal is the engagement of the antigen-specific T-cell receptor by the major histocompatibility complex (MHC) class II antigen–peptide complex. The second or costimulatory signal can vary from system to system, but for at least some lymphocytes, it is the binding of the B7 molecule to its cognate receptor, CD28, on the responding T cell (5–8). In this report we show that malignant tumor cells can be highly effective immunogens in the autologous host if they are engineered to present tumor antigen and deliver the B7 coactivation signal. Immunization with such engineered tumor cells generates potent tumor-specific CD4+ T cells that facilitate rejection and confer immunologic memory to high-dose challenges of wild-type neoplastic cells. These results demonstrate the critical role of the B7 costimulatory pathway in stimulating tumor-specific CD4+ T cells and provide an attractive strategy for enhancing tumor immunity.

## MATERIALS AND METHODS

**Cells.** SaI tumor cells were maintained as described (1).

**Antibodies.** The monoclonal antibody (mAb) 10-3.6, specific for I-$A^k$ (9), was prepared and used as described (1). The B7-specific mAb 1G10 is a rat IgG2a mAb and was used as described (10). mAbs specific for CD4+ [GK1.5 (11)] and CD8+ [2.43 (12)] were used as ascites fluid.

**Transfections.** Mouse SaI sarcoma cells were transfected as described (1) with wild-type $A\alpha^k$ and $A\beta^k$ MHC class II cDNAs, $A\alpha^k$ and $A\beta^k$ cDNAs truncated for their C-terminal 12 and 10 amino acids, respectively (13), and/or B7 gene (14). Class II transfectants were cotransfected with pSV2neo plasmid and selected for resistance to G418 (400 μg/ml). B7 transfectants were cotransfected with pSV2hph plasmid and selected for hygromycin-resistance (400 μg/ml). All transfectants were cloned twice by limiting dilution, except SaI/B7 transfectants, which were uncloned, and maintained in drug. Double transfectants were maintained in G418 plus hygromycin. The numbers after each transfectant are the clone designation.

**Immunofluorescence.** Indirect immunofluorescence was performed as described (1), and samples were analyzed on an Epics C flow cytometer.

**Tumor Challenges.** For primary tumor challenges, autologous A/J mice were challenged i.p. with the indicated number of tumor cells. Inoculated mice were checked three times per week for tumor growth. Mean survival times of mice dying from their tumor ranged from 13 to 28 days after inoculation. Mice were considered to have died from their tumor if they contained a large volume of ascites fluid and tumor cells (≥5 ml) at the time of death. Mice were considered tumor-resistant if they were tumor-free for at least 60 days after tumor challenge (range, 60–120 days). Tumor cells were monitored by indirect immunofluorescence for I-$A^k$ and B7 expression prior to tumor-cell inoculation. For the experiments of Table 2, autologous A/J mice were immunized i.p. with a single inoculum of the indicated number of live tumor cells and challenged i.p. with the indicated number of wild-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; APC, antigen-presenting cell; mAb, monoclonal antibody.

†To whom reprint requests should be addressed.

type SaI cells 42 days after immunization. Mice were evaluated for tumor resistance or susceptibility using the same criteria as for primary tumor challenge.

***In vivo* T-Cell Depletions.** A/J mice were depleted for $CD4^+$ or $CD8^+$ T cells by i.p. inoculation with 100 μl of ascites fluid of mAb GK1.5 ($CD4^+$ specific; ref. 11) or mAb 2.43 ($CD8^+$ specific; ref. 12) on days −6, −3, and −1 prior to tumor challenge, and every third day after tumor challenge as described (15) until the mice died or day 28, whichever came first. Presence or absence of tumor was assessed up to day 28. Previous studies have established that A/J mice with large tumors at day 28 after injection will progress to death. This time point was, therefore, chosen to assess tumor susceptibility for the *in vivo* depletion experiments. One mouse per group was sacrificed on day 28, and its spleen was assayed by immunofluorescence to ascertain depletion of the relevant T-cell population.

## RESULTS

**Coexpression of B7 Compensates for the Absence of the MHC Class II Cytoplasmic Domain and Restores Immunogenicity.** The mouse SaI sarcoma is an ascites-adapted class $I^+$ class $II^-$ tumor of A/J ($H\text{-}2K^kA^kD^d$) mice. The wild-type tumor is lethal in autologous A/J mice when administered i.p. SaI cells transfected with, and expressing, syngeneic MHC class II genes ($Aa^k$ and $Ab^k$ genes; SaI/$A^k$ cells) are immunologically rejected by the autologous host, and immunization with live SaI/$A^k$ cells protects mice against subsequent challenges with wild-type class $II^-$ SaI cells (1). Adoptive transfer (16) and lymphocyte depletion studies (E. Lamoussé-Smith and S.O.-R., unpublished data) demonstrate that SaI and SaI/$A^k$ rejection is dependent on $CD4^+$ lymphocytes. SaI cells expressing class II molecules with truncated cytoplasmic domains (SaI/$A^k$tr cells), however, are as lethal as wild-type class $II^-$ SaI cells, suggesting that the cytoplasmic region of the class II heterodimer is required to induce protective immunity (17).

It has recently been demonstrated that up-regulation of the B7 activation molecule on the APC is triggered by intracellular signals transmitted by the cytoplasmic domain of the class II heterodimer, after presentation of antigen to $CD4^+$ T helper cells (10). Inasmuch as B7 expression is normally up-regulated *in vivo* on SaI cells expressing full-length class II molecules (S.B. and S.O.-R., unpublished data), we have speculated that SaI/$A^k$tr cells do not stimulate protective immunity because they do not transmit a costimulatory signal.

To test whether B7 expression can compensate for the absence of the class II cytoplasmic domain, SaI/$A^k$tr cells were supertransfected with a plasmid containing a cDNA encoding murine B7 under the control of the cytomegalovirus promoter and screened for I-$A^k$ and B7 expression by indirect immunofluorescence. Wild-type SaI cells do not express either I-$A^k$ or B7 (Fig. 1 *a* and *b*), whereas SaI cells transfected with $Aa^k$ and $Ab^k$ genes (SaI/$A^k$ cells) or truncated $Aa^k$ and $Ab^k$ genes (SaI/$A^k$tr cells) express I-$A^k$ (Fig. 1 *d* and *f*) and do not express B7 (Fig. 1 *c* and *e*). SaI cells transfected with truncated class II genes plus the B7 gene (SaI/$A^k$tr/B7 cells) express I-$A^k$ and B7 molecules (Fig. 1 *g* and *h*). All cells express uniform levels of MHC class I molecules ($K^k$ and $D^d$) comparable to the level of I-$A^k$ in Fig. 1*h* (data not shown).

Antigen-presenting activity of the transfectants was tested by determining their immunogenicity and lethality in autologous A/J mice. As shown in Table 1, wild-type SaI cells administered i.p. at doses as low as $10^4$ cells are lethal in 88–100% of mice inoculated within 13–28 days after challenge, whereas 100 times as many SaI/$A^k$ cells are uniformly rejected. Challenges with similar quantities of SaI/$A^k$tr cells are also lethal; however, SaI/$A^k$tr cells that coexpress B7 (SaI/$A^k$tr/B7 clones -1 and -3) are uniformly rejected. A/J mice challenged with SaI/$A^k$tr cells transfected with the B7 construct, but not expressing detectable amounts of B7 antigen (SaI/$A^k$tr/hph cells), are as lethal as SaI/$A^k$tr cells, demonstrating that reversal of the malignant phenotype in SaI/$A^k$tr/B7 cells is due to expression of B7. SaI cells transfected with the B7 gene and not coexpressing truncated class II molecules (SaI/B7 cells, uncloned) are also as lethal as wild-type SaI cells, indicating that B7 expression without truncated class II molecules does not stimulate immunity. To

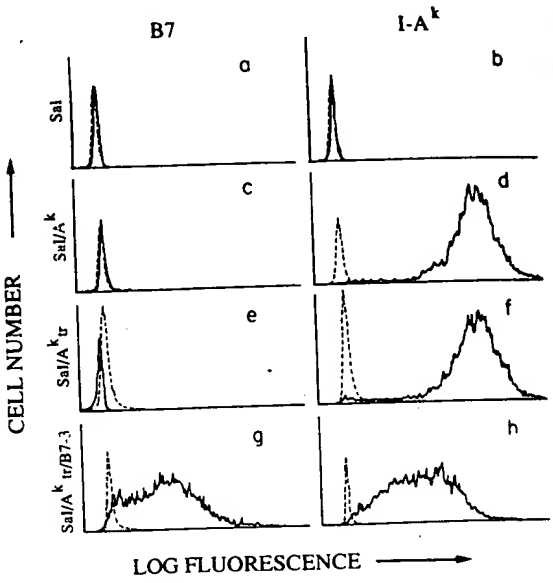


FIG. 1. SaI tumor cells transfected with *I-A^k* and B7 genes express these molecules at the cell surface. SaI/$A^k$, SaI cells transfected with wild-type $Aa^k$ and $Ab^k$ genes, clone 19.6.4; SaI/$A^k$tr, SaI cells transfected with truncated $Aa^k$ and $Ab^k$ genes, clone 6.11.8; SaI/$A^k$tr/B7, SaI cells transfected with truncated $Aa^k$ and $Ab^k$ genes and supertransfected with the B7 gene. All SaI/$A^k$tr/B7 clones tested consistently express lower levels of MHC class II antigen than SaI/$A^k$tr or SaI/$A^k$ cells. Abscissa represents three orders of magnitude of fluorescence intensity. Dotted lines represent control immunofluorescent staining by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (*b*, *d*, *f*, and *h*) or FITC-conjugated goat anti-rat immunoglobulin (*a*, *c*, *e*, and *g*); solid lines represent staining by I-$A^k$-specific mAb 10-3.6 (9) plus FITC-conjugated goat anti-mouse immunoglobulin (*b*, *d*, *f*, and *h*) or B7-specific mAb 1G10 (10) plus FITC-conjugated goat anti-rat immunoglobulin (*a*, *c*, *e*, and *g*).

Table 1. Tumorigenicity of B7 and MHC class II-transfected SaI tumor cells

| Challenge tumor | Expression I-$A^k$ | Expression B7 | Tumor dose, no. of cells | Mice dead/mice tested, no./no. |
|---|---|---|---|---|
| SaI | – | – | $1 \times 10^6$ | 9/10 |
| | – | – | $1 \times 10^5$ | 8/10 |
| | – | – | $1 \times 10^4$ | 7/8 |
| SaI/$A^k$ 19.6.4 | $A^k$ | – | $1 \times 10^6$ | 0/12 |
| | $A^k$ | – | $5 \times 10^5$ | 0/5 |
| | $A^k$ | – | $1 \times 10^5$ | 0/5 |
| SaI/$A^k$tr 6.11.8 | $A^k$tr | – | $1 \times 10^6$ | 12/12 |
| | $A^k$tr | – | $5 \times 10^5$ | 5/5 |
| | $A^k$tr | – | $1 \times 10^5$ | 5/10 |
| SaI/$A^k$tr/B7-1 | $A^k$tr | B7 | $1 \times 10^6$ | 0/4 |
| SaI/$A^k$tr/B7-3 | $A^k$tr | B7 | $1 \times 10^6$ | 0/5 |
| | $A^k$tr | B7 | $4 \times 10^5$ | 0/5 |
| | $A^k$tr | B7 | $1 \times 10^5$ | 0/5 |
| SaI/$A^k$tr/hph | $A^k$tr | – | $1 \times 10^6$ | 5/5 |
| SaI/B7 | – | B7 | $1 \times 10^6$ | 5/5 |

ascertain that rejection of SaI/A$^k$ and SaI/A$^k$tr/B7 cells is immunologically mediated, sublethally irradiated (900 rads; 1 rad = 0.01 Gy) A/J mice were challenged i.p. with these cells. In all cases, irradiated mice died from the tumor. We conclude that immunogenicity and host rejection of the MHC class II$^+$ tumor cells are dependent on an intact class II molecule and that coexpression of B7 can bypass the requirement for the class II intracellular domain.

**Immunization with B7-Transfected Sarcoma Cells Protects Against Later Challenges of Wild-Type B7$^-$ Sarcoma.** Activation of at least some T cells is thought to be dependent on coexpression of B7. However, once the T cells are activated, B7 expression is not required on the target cell for recognition by effector T cells. We have therefore tested the ability of three SaI/A$^k$tr/B7 clones (B7-3, B7-1, and B7-2B5.E2) to immunize A/J mice against subsequent challenges of wild-type class II$^-$ B7$^-$ SaI cells (Table 2). A/J mice were immunized with live SaI/A$^k$tr/B7 transfectants and 42 days later challenged with wild-type SaI tumor cells. Ninety-seven percent of mice immunized with the SaI/A$^k$tr/B7 transfectants were immune to $\geq 10^6$ wild-type B7$^-$ class II$^-$ SaI cells, an immunity that is comparable to that induced by immunization with SaI cells expressing full-length class II molecules. SaI/A$^k$tr/B7 cells, therefore, stimulate a potent response with long-term immunological memory against high-dose challenges of malignant tumor cells. B7 expression is, therefore, critical for the stimulation of SaI-specific effector cells; however, its expression is not needed on the tumor targets once the appropriate effector T-cell populations have been generated.

**Immunization with B7-Transfected Tumor Cells Stimulates Tumor-Specific CD4$^+$ Lymphocytes.** To ascertain that B7 is functioning through a T-cell pathway in tumor rejection, we have *in vivo*-depleted A/J mice for CD4$^+$ or CD8$^+$ T cells and challenged them i.p. with SaI/A$^k$ or SaI/A$^k$tr/B7 cells. As shown in Table 3, *in vivo* depletion of CD4$^+$ T cells results in host susceptibility to both SaI/A$^k$ and SaI/A$^k$tr/B7 tumors, indicating that CD4$^+$ T cells are critical for tumor rejection, whereas depletion of CD8$^+$ T cells does not affect SaI/A$^k$tr/B7 tumor rejection. Although immunofluorescence analysis of splenocytes of CD8$^+$-depleted mice demonstrates the absence of CD8$^+$ T cells, it is possible that the depleted mice contain small quantities of CD8$^+$ cells that are below our level of detection. These data therefore demonstrate that CD4$^+$ T cells are required for tumor rejection but do not eliminate a possible corequirement for CD8$^+$ T cells.

Previous adoptive transfer experiments (16) have demonstrated that both CD4$^+$ and CD8$^+$ T cells are required for rejection of class II$^-$ wild-type SaI cells. Inasmuch as rejection of SaI/A$^k$ and SaI/A$^k$tr/B7 cells appears to require only CD4$^+$ T cells, it is likely that immunization with class II$^+$ transfectants stimulates both CD4$^+$ and CD8$^+$ effector T cells; however, only the CD8$^+$ effectors are required for rejection of class I$^+$II$^-$ tumor targets. Costimulation by B7, therefore, enhances immunity by stimulating tumor-specific CD4$^+$ helper and cytotoxic lymphocytes.

Table 2. Autologous A/J mice immunized with SaI/A$^k$tr/B7 cells are immune to challenges of wild-type SaI tumor

| Immunization | No. of immunizing cells | SaI challenge dose, no. of cells | Mice dead/ mice tested, no./no. |
|---|---|---|---|
| None | — | $1 \times 10^6$ | 5/5 |
| SaI/A$^k$ 19.6.4 | $1 \times 10^5$ or $10^6$ | $1 \times 10^6$ | 0/5 |
| | $1 \times 10^6$ | $6 \times 10^6$ | 0/5 |
| SaI/A$^k$tr/B7-3 | $1 \times 10^6$ | $6 \times 10^6$ | 0/5 |
| | $1 \times 10^6$ | $1 \times 10^6$ | 0/5 |
| | $4 \times 10^5$ | $1 \times 10^6$ | 0/5 |
| | $1 \times 10^5$ | $5 \times 10^6$ | 0/5 |
| SaI/A$^k$tr/B7-1 | $5 \times 10^5$ | $3 \times 10^6$ | 0/3 |
| | $2 \times 10^5$ | $1 \times 10^6$ | 0/2 |
| | $5 \times 10^4$ | $5 \times 10^6$ | 0/3 |
| SaI/A$^k$tr/B7-2B5.E2 | $1 \times 10^5$ | $2 \times 10^5$ | 0/2 |
| | $5 \times 10^4$ | $2 \times 10^6$ | 1/7 |

Table 3. Tumor susceptibility of A/J mice *in vivo*-depleted for CD4$^+$ or CD8$^+$ T cells

| Tumor challenge | Host T-cell depletion | No. mice with tumor/ total no. mice challenged |
|---|---|---|
| SaI/A$^k$ | CD4$^+$ | 3/5 |
| SaI/A$^k$tr/B7-3 | CD4$^+$ | 5/5 |
| | CD8$^+$ | 0/5 |

## DISCUSSION

In other recent studies, we have shown (18) that SaI/A$^k$ cells supertransfected with the class II-associated invariant chain gene (Ii) are as malignant as wild-type SaI cells, indicating that class II$^+$ tumor cells that coexpress Ii are unable to stimulate tumor-specific immunity. Inasmuch as Ii is thought to inhibit the presentation of endogenously synthesized peptides by class II molecules (19–26), these data suggest that the increased immunogenicity of SaI/A$^k$ cells is due to the presentation of endogenously synthesized tumor peptides. Collectively, these data are consistent with the hypothesis that Ii$^-$ SaI/A$^k$ cells stimulate potent tumor-specific immunity because their class II molecules directly present endogenously synthesized tumor peptides to CD4$^+$ T cells, thereby improving the generation of tumor-specific T helper cells. The ability of the class II$^+$ tumor cells to directly present tumor peptides to CD4$^+$ T helper cells bypasses the need for third-party APCs and probably improves tumor immunogenicity because soluble tumor antigen (in the form of tumor-cell debris or secreted protein) may not be available for uptake by professional APCs.

Inasmuch as rejection of the SaI sarcoma by autologous A/J mice is T-cell-mediated, these results support the two-signal model for T-cell activation in primary immune responses. Previous studies have established the requirement for a second signal for activation of T cells *in vitro* (5–8); however, the present results document the requirement for both first and second signals for effective T-cell activation within the complex *in vivo* setting of autologous tumor rejection.

The requirement for a costimulatory signal for generation of effective tumor-specific immunity raises the question of whether inadequate anti-tumor responses are due to insufficient generation of a first or second signal. Indeed, in the absence of costimulation, tumor-specific T cells may be anergized, leading to tolerance (4). This scenario may occur in malignant disease if tumor-cell debris is not present or if tumor antigens are not secreted, and hence, tumor peptides are not available for uptake by APCs that constitutively express costimulatory molecules such as the B7 activation antigen.

Although SaI is a weakly immunogenic tumor, it can induce effective tumor-specific immunity if, by transfection, it expresses the appropriate antigen-presenting elements (i.e., MHC class II molecules) and delivers the required signals (e.g., B7) to responding T cells. The inability of the autologous host to respond to wild-type tumor cells is, therefore, probably not due to lack of expression of tumor peptides but rather to inadequate presentation of these peptides and/or to delivery of the required additional activation signals.

Two other reports (27, 28) have also demonstrated the efficacy of B7 expression for improving tumor-specific immunity; however, two important differences distinguish the present report from these studies. In both of the previous studies, the K1735 mouse melanoma was transfected with the B7 gene. Interestingly, Chen *et al.* (27) cotransfected with the E7 viral gene from human papillomavirus, and the resulting immunity was specific for and dependent on expression of the E7 gene product. Inasmuch as $E7^-$ melanoma cells were not targets for B7-stimulated effectors, this study suggested that constitutive B7 expression would not be applicable as immunotherapy for wild-type established tumors. In the Townsend and Allison study (28), however, using the same K1735 tumor, coexpression of a viral antigen was not required for immunity. Likewise, in our study, expression of a xenogeneic tumor antigen is not required, and immunity appears to be directed against endogenously encoded murine tumor molecules. Hence, our studies support the contention that coexpression of B7 can stimulate potent immunity to natural tumor antigens and, therefore, provide a strong experimental basis for stimulating immunity to spontaneous resident malignancies.

In the present report, we demonstrate that B7-transfected sarcoma cells stimulate potent tumor-specific $CD4^+$ effector cells, whereas in the studies of Chen *et al.* (27) and Townsend and Allison (28), immunization with B7-transfected melanoma cells induced $CD8^+$ effectors. This difference in effector population is probably the result of the presentation of tumor peptide by different MHC gene products. In the K1735 melanoma system, the tumor antigen is most likely presented by MHC class I molecules, whereas in our sarcoma system tumor peptide is presented by MHC class II molecules. Collectively, the three studies demonstrate that under the appropriate conditions, coexpression of B7 can optimize stimulation of both $CD4^+$ and $CD8^+$ T cells, thereby enhancing the tumor-specific immune response in both T-cell compartments.

In the experimental system described in this report, constitutive expression of B7 appears to provide the costimulatory signal for T-cell activation in the absence of the MHC class II cytoplasmic domain. Aside from being a formal demonstration of the role of the class II cytoplasmic domain in second signal induction, this result provides an experimental framework for improving tumor-specific immunity. Our previous approach for improving tumor-specific responses has been to constitutively express syngeneic MHC class II molecules in tumor cells (1) and rely on the transient induction of costimulatory signals during the immunization process. However, a wider repertoire of tumor-specific T cells may be activated, resulting in a more potent primary response, if B7 is stably expressed by the class $II^+$ tumor. Tumor cells stably coexpressing B7 and syngeneic MHC class II molecules may, therefore, be very useful immunogens for protecting against subsequent metastatic disease and/or for rescuing individuals carrying established tumors.

We thank Virginia K. Clements for her outstanding technical help, Sandy Mason for her excellent care of our mouse colony, and Dr. H. Auchincloss for providing the GK1.5 and 2.43 ascites fluid. These studies were supported by National Institutes of Health grants to S.O.-R. (R01CA52527) and L.H.G. (R01AI21569) and Elsa Pardee Foundation and Maryland American Cancer Society Institutional grants to S.O.-R.

1. Ostrand-Rosenberg, S., Thakur, A. & Clements, V. (1990) *J. Immunol.* **144,** 4068–4071.
2. Schultz, K., Klarnet, J., Gieni, R., Hayglass, K. & Greenberg, P. (1990) *Science* **249,** 921–923.
3. Fearon, E. R., Pardoll, D., Itaya, T. & Golumbek, P. (1990) *Cell* **60,** 397–403.
4. Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. (1989) *Annu. Rev. Immunol.* **7,** 445–480.
5. Gimmi, C. D., Freeman, G. J., Gribben, J. G., Sugita, K., Freedman, A. S., Morimoto, C. & Nadler, L. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 6575–6579.
6. Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. & Ledbetter, J. (1991) *J. Exp. Med.* **173,** 721–730.
7. Koulova, L., Clark, E. A., Shu, G. & Dupont, B. (1991) *J. Exp. Med.* **173,** 759–762.
8. Razi-Wolf, Z., Freeman, G. J., Galvin, F., Benacerraf, B., Nadler, L. & Reiser, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 4210–4214.
9. Oi, V., Jones, P., Goding, J., Herzenberg, L. & Herzenberg, L. (1978) *Curr. Top. Microbiol. Immunol.* **81,** 115–120.
10. Nabavi, N., Freeman, G. J., Gault, A., Godfrey, D., Nadler, L. M. & Glimcher, L. H. (1992) *Nature (London)* **360,** 266–268.
11. Wilde, D. B., Marrack, P., Kappler, J., Dialynas, D. D. & Fitch, F. W. (1983) *J. Immunol.* **131,** 2178–2183.
12. Sarmiento, M., Glasebrook, A. L. & Fitch, F. W. (1980) *J. Immunol.* **125,** 2665–2672.
13. Nabavi, N., Ghogawala, Z., Myer, A., Griffith, I., Wade, W., Chen, Z., McKean, D. & Glimcher, L. (1989) *J. Immunol.* **142,** 1444–1447.
14. Freeman, G., Gray, G., Gimmi, C., Lombard, D., Zhou, L., White, M., Fingeroth, J., Gribben, J. & Nadler, L. (1991) *J. Exp. Med.* **174,** 625–631.
15. Ghobrial, M., Boublik, M., Winn, H. J. & Auchincloss, H., Jr. (1989) *Clin. Immunol. Immunopathol.* **52,** 486–506.
16. Cole, G. & Ostrand-Rosenberg, S. (1991) *Cell. Immunol.* **134,** 480–490.
17. Ostrand-Rosenberg, S., Roby, C. & Clements, V. K. (1991) *J. Immunol.* **147,** 2419–2422.
18. Clements, V. K., Baskar, S., Armstrong, T. & Ostrand-Rosenberg, S. (1992) *J. Immunol.* **149,** 2391–2396.
19. Elliott, W., Stile, C., Thomas, L. & Humphrey, R. (1987) *J. Immunol.* **138,** 2949–2952.
20. Stockinger, B., Pessara, U., Lin, R., Habicht, J., Grez, M. & Koch, N. (1989) *Cell* **56,** 683–689.
21. Guagliardi, L., Koppelman, B., Blum, J., Marks, M., Cresswell, P. & Brodsky, F. (1990) *Nature (London)* **343,** 133–139.
22. Bakke, O. & Dobberstein, B. (1990) *Cell* **63,** 707–716.
23. Lotteau, V., Teyton, L., Pelerauz, A., Nilsson, T., Karlsson, L., Schmid, S., Quaranta, V. & Peterson, P. (1990) *Nature (London)* **348,** 600–605.
24. Peters, J., Neefjes, J., Oorschot, V., Ploegh, H. & Geuze, H. (1991) *Nature (London)* **349,** 669–676.
25. Roche, P. & Cresswell, P. (1990) *Nature (London)* **345,** 615–618.
26. Teyton, L., O'Sullivan, D., Dickson, P., Lotteau, V., Sette, A., Fink, P. & Peterson, P. (1990) *Nature (London)* **348,** 39–44.
27. Chen, L., Ashe, S., Brady, W., Hellstrom, I., Hellstrom, K., Ledbetter, J., McGowan, P. & Linsley, P. (1992) *Cell* **71,** 1093–1102.
28. Townsend, S. E. & Allison, J. P. (1993) *Science* **259,** 368–370.

# Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies

Y M Deo, R F Graziano, R Repp and J G J van de Winkel




Elsevier Trends Journals
68 Hills Road
Cambridge
UK CB2 1LA
Tel +44 1223 315961
Fax +44 1223 321410

# Clinical significance of IgG Fc receptors and FcγR-directed immunotherapies

**Yashwant M. Deo, Robert F. Graziano, Roland Repp and Jan G.J. van de Winkel**

*Fc receptors for IgG (FcγRs) can trigger the inflammatory, cytotoxic and hypersensitivity functions of immune effector cells. Activation or deactivation of effector cells via FcγRs can be exploited to develop novel therapies for cancer, infectious diseases and autoimmune disorders. Initial results of clinical trials for several FcγR-directed immunotherapies show the potential promise of this approach.*

The IgG Fc receptors (FcγRs) are expressed primarily on immune effector cells, and link cellular and humoral immunity by serving as a bridge between antibody specificity and effector cell function. In this fashion, FcγRs act as trigger molecules for inflammatory, cytolytic, allergic (hypersensitivity), endocytic and phagocytic activities of immune effector cells. Moreover, since many FcγR-bearing cells are also antigen-presenting cells (APCs; e.g. macrophages, dendritic cells), FcγR-mediated internalization via phagocytosis may also lead to antigen presentation and amplification of the immune response. These functions of FcγRs are linked to activation and regulation of immune defense in various disease conditions. The position of FcγRs as a gateway both to cellular and humoral aspects of the immune cascade makes them potentially attractive candidates for directed immunotherapy. This review focuses on the clinical significance of FcγRs and developments in FcγR-directed therapies for cancer, infectious diseases and autoimmune disorders.

## FcγR structure and function

There are three classes of FcγR: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). These classes comprise nine membrane-associated and three soluble FcγR molecules, encoded by eight genes (Fig. 1a). FcγRs are expressed by most hematopoietic cells, and their expression can be enhanced by certain inflammatory cytokines such as interferon γ (IFN-γ) and granulocyte colony-stimulating factor (G-CSF) (Table 1)[1,2]. With the exception of the glycosylphosphatidylinositol (GPI)-linked FcγRIIIb, all FcγRs are transmembrane molecules belonging to the family of multichain immune recognition receptors (MIRRs), which also includes the B-cell receptor (BCR) and T-cell receptor (TCR). FcγRIa is a high-affinity receptor and contains three Ig-like domains in its extracellular region, instead of two as in all other FcγRs. FcγRII and FcγRIII represent low-affinity receptors. Most FcγRs exist as hetero-oligomeric complexes with a ligand-binding α-chain and a signaling component comprising γ-, β- or ζ-chains (Fig. 1a, Table 1). Each signaling chain bears a unique ~26 amino acid immunoreceptor tyrosine-based activation motif (ITAM) involved in activatory functions. A similar, albeit noncanonical, ITAM is located in the cytoplasmic region of FcγRIIa and appears to be critical for cell activation by this receptor[1]. Recently, FcγRIIa has been shown to be capable of interacting with the FcR γ-chain, which modulates its signaling behavior[3,4]. The FcγRIIb members contain a unique 13 amino acid immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain, and this is important in immune-inhibitory functions. Conserved tyrosine and leucine residues within these signaling motifs play a central role in FcγR signal transduction[5].

Additional FcγR heterogeneity is introduced by polymorphisms (Fig. 1b). The myeloid FcγRIIa (CD32) differs by a single amino acid within the second Ig-like domain, either an arginine or histidine at position 131 (FcγRIIa-R131 or FcγRIIa-H131)[6]. The neutrophil FcγRIIIb-NA1 and -NA2 allotypes differ by five nucleotides, which result in an increased number of glycosylation sites in FcγRIIIb-NA2 (six versus four)[7]. In addition, amino acid variation at position 48 distinguishes three allotypes of FcγRIIIa (Ref. 8). Furthermore, amino acid variation at position 158 of FcγRIIIa results in a polymorphism with functional consequences (H.R. Koene *et al.*, unpublished).

Although the extracellular domains of various FcγRs do not exhibit exclusive specificity for ligands (Table 1), individual FcγRs trigger characteristic biological responses determined by both the nature of the effector cell and the transmembrane and cytoplasmic regions of the receptor[1,2]. Furthermore, the transmembrane domains of MIRRs may functionally interact. For example: on neutrophils, crosslinking of FcγRIIIb enhances FcγRIIa-mediated phagocytosis[9]; on B cells, co-crosslinking of FcγRIIb and the BCR results in down-modulation of antibody secretion; and, on neutrophils, complement receptor 3 (CR3; CD11b/CD18) acts as a signaling partner for GPI-linked FcγRIIIb (Refs 5, 10, 11). The first step in FcγR activation is receptor crosslinking, with as few as two crosslinked receptors activating the signaling cascade (Fig. 2). Crosslinking at the FcγR ligand-binding domain, as well as outside this domain [via anti-receptor monoclonal antibodies (mAbs)], triggers FcγR function[1,12]. The second step involves phosphorylation of tyrosine residues

PII: S0167-5699(97)01067-4

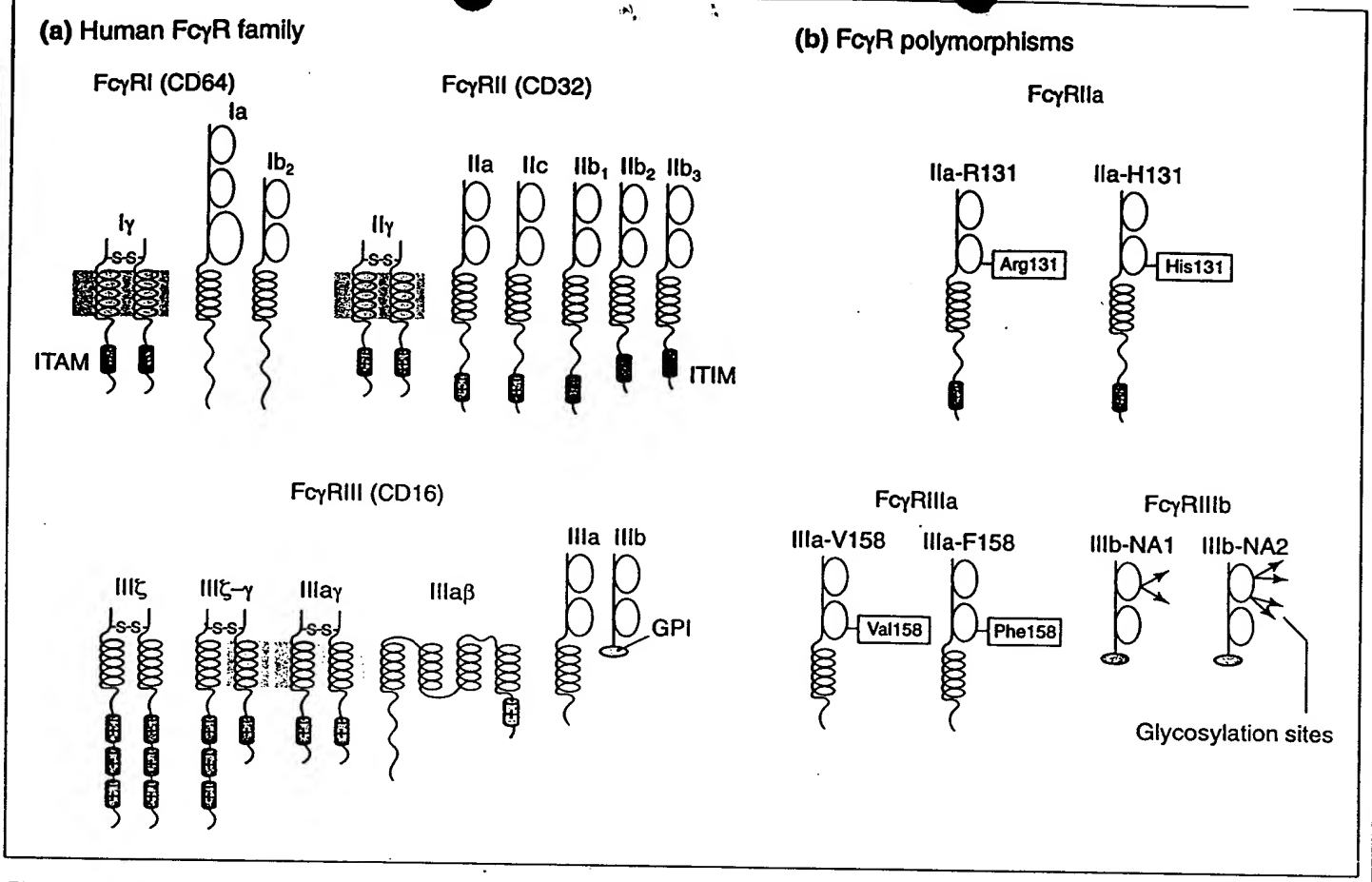


**Fig. 1.** *(a) The human FcγR family. The ligand-binding α-chains of all receptors contain extracellular regions comprising disulfide-bonded immunoglobulin (Ig)-like domains. FcγRI has three Ig-like domains, the others have two Ig-like domains. FcγRIa, FcγRIIa and FcγRIIIa exist as oligomeric complexes with associated FcR γ-, β- or ζ-chains, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) indicated by the plus sign. FcγRIIb molecules contain an inhibitory motif (ITIM), indicated by the minus sign. All three classes contain soluble molecules not shown in this diagram. (b) FcγR polymorphisms. Two allotypic forms of human FcγRIIa have been distinguished by the presence of either arginine (FcγRIIa-R131) or histidine (FcγRIIa-H131) at position 131. The two allotypes of FcγRIIIa contain either valine or phenylalanine at position 158. The FcγRIIIb-NA1 and -NA2 allotypes differ by five nucleotides, which results in differential glycosylation (indicated by the arrow heads). Abbreviation: GPI, glycosylphosphatidylinositol.*

within the ITAM of FcγRs by *src*-family protein tyrosine kinases (PTKs). This is followed by association and activation of *syk*-family PTKs with the phosphorylated ITAM. The subsequent events are not clearly delineated but appear to involve several distinct signaling components leading to different biological responses[1].

FcγR-expressing cells activated via these signaling cascades are able to lyse or phagocytose IgG-opsonized pathogens or tumor cells, as well as clear immune complexes (ICs), promote antigen presentation and induce inflammation. The FcγR-dependent phagocytic and cytolytic [antibody-dependent cellular cytotoxicity (ADCC)] activities are well documented. These activities play a key role in immune defense against infectious diseases, and probably in immune surveillance against malignant cell growth. *In vitro*, targeting antigens to FcγRs on macrophages and dendritic cells significantly facilitates antigen presentation[13]. Similar data have been obtained in a human FcγRI (huFcγRI) transgenic mouse model in which the transgenic animals induced a much greater humoral response to FcγRI-directed antigens than the nontransgenic littermates, supporting a role for huFcγRI in antigen presentation[14]. In the same model, a role for FcγRI in inflammatory processes was suggested by a dramatic increase in phagocyte expression of huFcγRI in mice with inflammatory lesions. In contrast to these immune defense functions, activation of FcγR by autoantibodies or defects in FcγR functions are implicated in several autoimmune disorders. Recently, the significance of FcγRs in type II and III hypersensitivity reactions has been firmly established by defective anaphylactic and inflammatory responses observed in mice deficient in the FcR γ-chain or in FcγRIII (Refs 15–17). Thus, the pleiotropic biological responses induced via FcγRs play a significant role in various diseases. Therefore, therapies that harness these cytotoxic and immune activation functions of FcγRs, or downmodulate FcγR activity, are currently being developed.

## FcγRs and cancer

Destruction of tumor cells by FcγR-expressing effectors via ADCC and phagocytosis has been well established. Tumor-specific antibodies and bispecific molecules (BSMs) directed to FcγR-expressing effector cells represent two approaches developed to harness FcγR activities for cancer therapy. FcγR-directed tumor vaccines are also

being developed, since antigens directed to FcγRs on APCs induce strong antigen-specific immune activation[13,14].

### Role of FcγRs in antibody therapy

Tumor-specific mAbs can mediate destruction of tumor cells by phagocytosis or ADCC induced via binding to FcγRs. *In vitro* studies have shown mAb-mediated ADCC of a broad spectrum of tumor cell lines, derived both from solid tumors and hemato-lymphatic tumors, by FcγR-expressing monocytes, macrophages, eosinophils, neutrophils and natural killer (NK) cells[18,19]. Involvement of FcγRs in mAb-mediated cytotoxicity is supported by the following observations: (1) crosslinking FcγRs triggers cytotoxicity of specific immune effector cells; (2) serum IgG, which can compete with tumor-specific mAbs of certain isotypes for binding to FcγRs, inhibits mAb-mediated ADCC of tumor cells; (3) mAb-mediated tumoricidal activity of specific effector cells can be induced or enhanced by cytokines that upregulate FcγR expression[20]; (4) antitumor activity of different isotypes correlates with the ability of an isotype to engage FcγRs on cytotoxic effector cells; and (5) with a few exceptions, $F(ab')_2$ fragments of tumor-specific mAbs are ineffective in tumor cell killing.

*In vivo* studies in mouse models and clinical trials further support the *in vitro* observations. First, tumor-specific mAbs have been found to be equally effective in eradicating tumors in mice deficient in complement component C5 as in control mice, which thereby excludes complement-mediated tumor cell lysis in this model[21]. Furthermore, the capacity of antibodies to elicit tumor regression has been shown in certain cases to depend on FcγR-expressing effector cells[22]. Indeed, the rate of tumor rejection correlates with the density of FcγR-expressing effector cell infiltration at the tumor site following antibody therapy, and depletion of $FcR^+$ effector cells was found to abrogate mAb efficacy[22,23]. In addition, comparison of antibodies with the same tumor specificity but different isotypes shows a correlation between the capacity of an antibody to induce ADCC *in vitro* and its efficacy *in vivo* in mouse models[24]. In a clinical trial comparing isotype switch variants of CAMPATH antibody (specific for CDw52), the strongest depletion of malignant cells was observed with the antibody isotype that most effectively induced ADCC *in vitro*[18]. Adjuvant therapy with a murine IgG2a (a potent mediator of ADCC) tumor-specific mAb (anti-17-1A) reduced the overall death rate by >30% in colorectal cancer patients[25]. Human IgG1 has the broadest spectrum reactivity with human FcγRs (Table 1) and is, therefore, regarded as optimal for effector cell recruitment. In accordance, a humanized IgG1 anti-HER-2/*neu* antibody and a chimeric IgG1 anti-CD20 antibody have shown very encouraging clinical responses, emphasizing the importance of the human Fc region[19,26]. These studies indicate that the cytotoxic activity of FcγR-expressing effector cells may play an important role in the antitumor effects of tumor-reactive mAbs.

**Table 1 General characteristics of human leukocyte IgG receptors**

| | FcγRI (CD64) | FcγRII (CD32) | FcγRIII (CD16) |
|---|---|---|---|
| Molecular mass | 72 kDa | 40 kDa | 50–80 kDa |
| Affinity for IgG ($K_a$) | High ($10^8$–$10^9$ $M^{-1}$) | Low (<$10^7$ $M^{-1}$) | IIIa: medium (~$3 \times 10^7$ $M^{-1}$)<br>IIb: low (<$10^7$ $M^{-1}$) |
| Receptor subunits | γ chain | IIa: γ chain | IIIa: γ chain, ζ chain<br>IIb: β chain |
| Distribution | | | |
| Constitutive | CD34+ myeloid progenitors, monocytes, macrophages, dendritic cells | Monocytes, macrophages, B cells, platelets, basophils, neutrophils, Langerhans cells, eosinophils, endothelial cells (subpopulation), T cells (subpopulation), dendritic cells | IIIa: macrophages, NK cells, monocytes (subpopulation), T cells (subpopulation)<br>IIb: neutrophils |
| Induced | Neutrophils (IFN-γ, G-CSF) | | IIIa: monocytes (TGF-β) |
| Modulation | ↑: G-CSF, IFN-γ, IL-10<br>↓: IL-4, IL-13 | ↓: IL-4 | ↓: IL-4<br>↑: TGF-β |
| Affinity: | | | |
| Murine IgG | 2a=3>>>2b | 2b>>2a | 2a>2b>>1 |
| Human IgG | 3>1>4>>>2 | 3>1 | 1=3>>>2,4 |

Abbreviations: G-CSF, granulocyte colony-stimulating factor; IFN-γ, interferon γ; IL-4, interleukin 4; NK, natural killer; TGF-β, transforming growth factor β.
The three subclasses of FcγRI have distinct affinities for human IgG: FcγRIIa-R131, 3>1>>2; FcγRIIb1, 3>1>>4>>2.

### BSMs

In order to improve effector cell recruitment and FcγR activation at tumor sites, BSMs that have one arm specific for tumor cells and the other specific for FcγRs on immune effector cells have been developed[12]. These BSMs offer several advantages over conventional mAbs as detailed in Box 1.

FcγRI and FcγRIII are of particular interest for BSM targeting. FcγRI is expressed solely on cytotoxic effector cells and is always capable of triggering cytotoxic activity. Since it is typically saturated

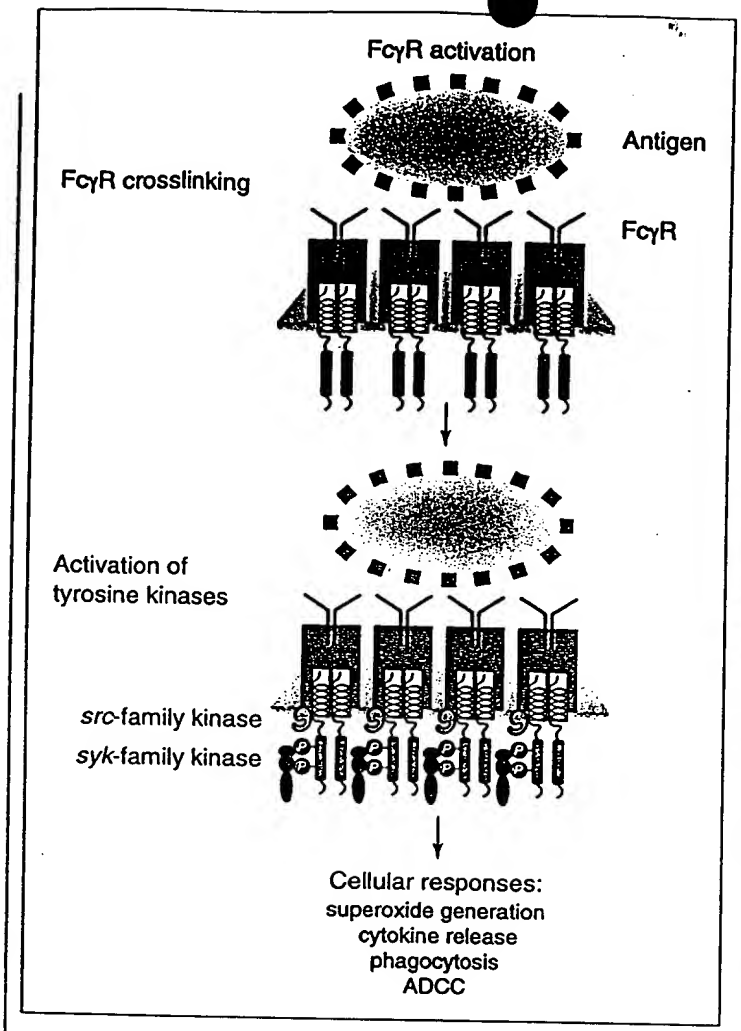


**Fig. 2.** *Schematic representation of effector cell activation through FcγRs. The crucial first step is crosslinking of FcγRs, and this is promoted by simultaneous binding of several antigen–IgG immune complexes to the extracellular region of FcγR α-chains. This results in the association and activation of* src*-family PTKs, inducing tyrosine phosphorylation (P) of the FcγR ITAM. This phosphorylation results in binding and activation of* syk*-family PTKs, followed by a cascade of events culminating in physiological responses. The exact point(s) of interaction between the PTKs and FcγRs has not been well established. Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ITAM, immunorecepter tyrosine-based activation motif; PTK, protein tyrosine kinase.*

with serum IgG under normal physiological conditions, it can be most effectively triggered to induce ADCC, phagocytosis and other effector functions by BSMs that can bind outside the Fc ligand-binding domain. Several such BSMs have been developed, with one arm specific for FcγRI and the other arm specific for a tumor marker such as CD15, HER-2/*neu*, epidermal growth factor receptor (EGFR)[57], disialoganglioside (GD2), HLA-DR (Ref. 27), CD19, CD37, or gastrin-releasing peptide (GRP) receptor (reviewed in Ref. 12). These BSMs readily direct monocytes, macrophages and IFN-γ- or G-CSF-activated polymorphonuclear leukocytes (PMNs) to tumor cells, and have proven to be highly effective in mediating ADCC and phagocytosis of tumor targets. FcγRIII is an important Fc receptor triggering ADCC by NK cells and it is also functional on macrophages (Table 1). BSMs specific for FcγRIII and tumor antigens

**Box 1. Advantages of bispecific molecules (BSMs)**

- BSMs are relatively small (50–100 kDa) and may penetrate tumors better than monoclonal antibodies (mAbs) (150–1000 kDa)
- BSMs can efficiently mediate effector cell (macrophage, neutrophil or natural killer (NK) cell)-dependent lysis of monolayers or spheroids of tumor cells
- BSMs can be constructed with or without an Fc region to retain or eliminate complement-activating capacity
- BSMs can be designed selectively to trigger FcγRs expressed solely on cytotoxic effector cells (e.g. FcγRI) to avoid triggering of noncytotoxic cells (e.g. platelets or B cells that express FcγRII)
- BSMs can be configured to bind to an epitope on FcγRs outside the Fc-binding domain to circumvent competition by serum IgG for FcγR binding and to maintain antitumor activity in the physiological environment
- BSMs can be devised to target specifically FcγRs on phagocytic cells, which function both as cytotoxic effectors and antigen-presenting cells, to promote tumor destruction and tumor-specific immunity
- BSMs do not require binding to tumor cells in order to engage Fc receptors, therefore effector cells may be 'armed' with BSMs, conferring on them specific antitumor activity

such as HER-2/*neu*, CD30, CA19-9, CD33 and high-molecular-weight melanoma antigen have shown effective killing of tumor cells *in vitro*. Efficacy of BSMs *in vivo* has been demonstrated in severe combined immunodeficiency (SCID) mice xenografted with human tumors. BSMs in combination with human effector cells induced long-term survival[28] and complete regression of established tumors[29].

Three BSMs directed to FcγRI and two directed to FcγRIII are currently being tested in clinical trials, either alone or in combination with cytokines that may enhance their efficacy (Table 2). Several phase I/II studies are under way with two BSMs (MDX-210 and MDX-447) comprising chemically linked F(ab') fragments of FcγRI- and HER-2/*neu*- or EGFR-specific antibodies, in late-stage cancer patients with various HER-2/*neu*$^+$ or EGFR$^+$ malignancies. Single and multiple doses (up to 25 mg m$^{-2}$) of BSMs are tolerated well, and induce immunological and biological responses[30,31]. After infusion, BSMs bind rapidly to FcγRI-expressing effector cells, and trigger both a transient disappearance of these cells from the circulation and a significant rise in serum levels of the inflammatory cytokines, tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and G-CSF. BSM-coated effector cells infiltrate tumors, resulting in tumor inflammation, tumor regression, a decrease in levels of tumor antigen in circulation, and improvement in symptomatic relief[30,31]. In some instances, up to 20-fold increases in serum levels of human antitumor antibodies (IgM and IgG) were observed, indicating that FcγRI-directed BSMs promote antigen presentation and induction of antitumor immune responses *in vivo* (P. Guyre *et al.*, unpublished). In another trial, a BSM comprising a mAb to CD15 linked with an FcγRI mAb was tested in

**Table 2. Clinical applications of FcγR-directed therapies**

| Disease and therapy | Status | Indication | Ref. |
|---|---|---|---|
| **Cancer** | | | |
| Antitumor antibodies | Phase I, II, III, approved | Various malignancies including: | |
| | | breast cancer | 26 |
| | | colorectal cancer | 25 |
| | | lymphoma | 19 |
| | | leukemia | 56 |
| Anti-CD15 × anti-FcγRI BSM | Phase I | Leukemia | 32 |
| Anti-HER-2/*neu* × anti-FcγRI BSM (MDX-210 with/without cytokines) | Phase I/II | HER-2/*neu*-overexpressing malignancies | 30, 31[a] |
| Anti-EGFR × anti-FcγRI BSM (MDX-447 with/without G-CSF) | Phase I | EGFR overexpressing malignancies | 5 |
| Anti-HER-2/*neu* × anti-FcγRII BSM (2B1 without cytokines) | Phase I/II | HER-2/*neu*-overexpressing malignancies | 33 |
| Anti-CD30 × anti-FcγRII BSM | Phase I/II | Hodgkin's lymphoma | |
| **Autoimmune disorders** | | | |
| Anti-FcγRIII antibody (3G8) | Phase I | ITP | 53 |
| Anti-FcγRI antibody (197) | Phase I | ITP | 54 |
| IgG Fc fragment | Phase I/II | ITP | 50 |
| Anti-Rh antibodies (WinRho) | Approved | ITP | 49 |
| **Infectious disease** | | | |
| Anti-gp41 × anti-FcγRI BSM (MDX-240) | Phase I | HIV seropositive | d |

Abbreviations: BSM, bispecific molecule; EGFR, epidermal growth factor receptor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte–monocyte colony-stimulating factor; HIV, human immunodeficiency virus; ITP, idiopathic thrombocytopenia purpura; Rh, Rhesus factor.
[a] J. Weber, L. Spears, V. Deo *et al.*, unpublished; J. Posey, V. Verma, J.L. Marshall *et al.*, unpublished.
[b] D. Pfister *et al.*, unpublished.
[c] F. Hartmann *et al.*, unpublished.
[d] J.L. Pasqualini *et al.*, unpublished.

four patients, one of which showed a transient decrease in leukemic cells[32]. A BSM specific for FcγRIII and CD30 has been tested in patients with Hodgkin's disease and shown to be tolerated well and able to elicit a clinical response in some patients (F. Hartmann *et al.*, unpublished). A BSM (2B1) specific for FcγRIII and HER-2/*neu*, and comprising a hetero-antibody containing the murine IgG1 Fc region, has been tested in HER-2/*neu*$^+$ patients[33]. Multiple doses of 2B1 induced elevated serum levels of TNF-α, IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF) and IFN-γ, as well as minor clinical responses. Notably, several patients exhibited significant increases in human anti-HER-2/*neu* antibodies of both IgM and IgG isotypes, indicating that 2B1 treatment induced specific antitumor immune cascades (J. Gralow *et al.*, unpublished). These encouraging results from clinical trials point to the potential promise of FcγR-directed BSMs in cancer therapy.

## FcγRs and infectious diseases

FcγRs are of crucial importance in directing the uptake and destruction of viruses, bacteria and a variety of infectious parasites, and are involved in antibody-dependent killing of infected cells expressing viral antigens[1,12]. FcγRIIIa-expressing NK cells isolated from human immunodeficiency virus (HIV)-seropositive individuals have been shown to be coated with anti-HIV antibodies and readily mediate lysis of HIV-infected or gp120-coated target cells *in vitro*. Furthermore, this ADCC activity correlates inversely with disease progression[34]. The importance of appropriate detection of IgG-opsonized microorganisms by FcγRs on phagocytes is further emphasized by susceptibility of individuals expressing the FcγRIIa-R131 allotype to infections by encapsulated bacteria. The FcγRIIa-H131 allotype (as opposed to FcγRIIa-R131) is identified as the only FcγR capable of binding human IgG2 (Ref. 6), an important isotype in immune defense against encapsulated bacteria. Neutrophils from individuals expressing the FcγRIIa-R131 allotype inefficiently phagocytose human IgG2-coated bacteria[35], rendering these individuals more susceptible to infection. Allotypic forms of FcγRIIIb (NA1 versus NA2) have also demonstrated differences in the binding and phagocytosis of IgG1- and IgG3-coated particles[36], which may have clinical relevance with regard to susceptibility to infectious disease.

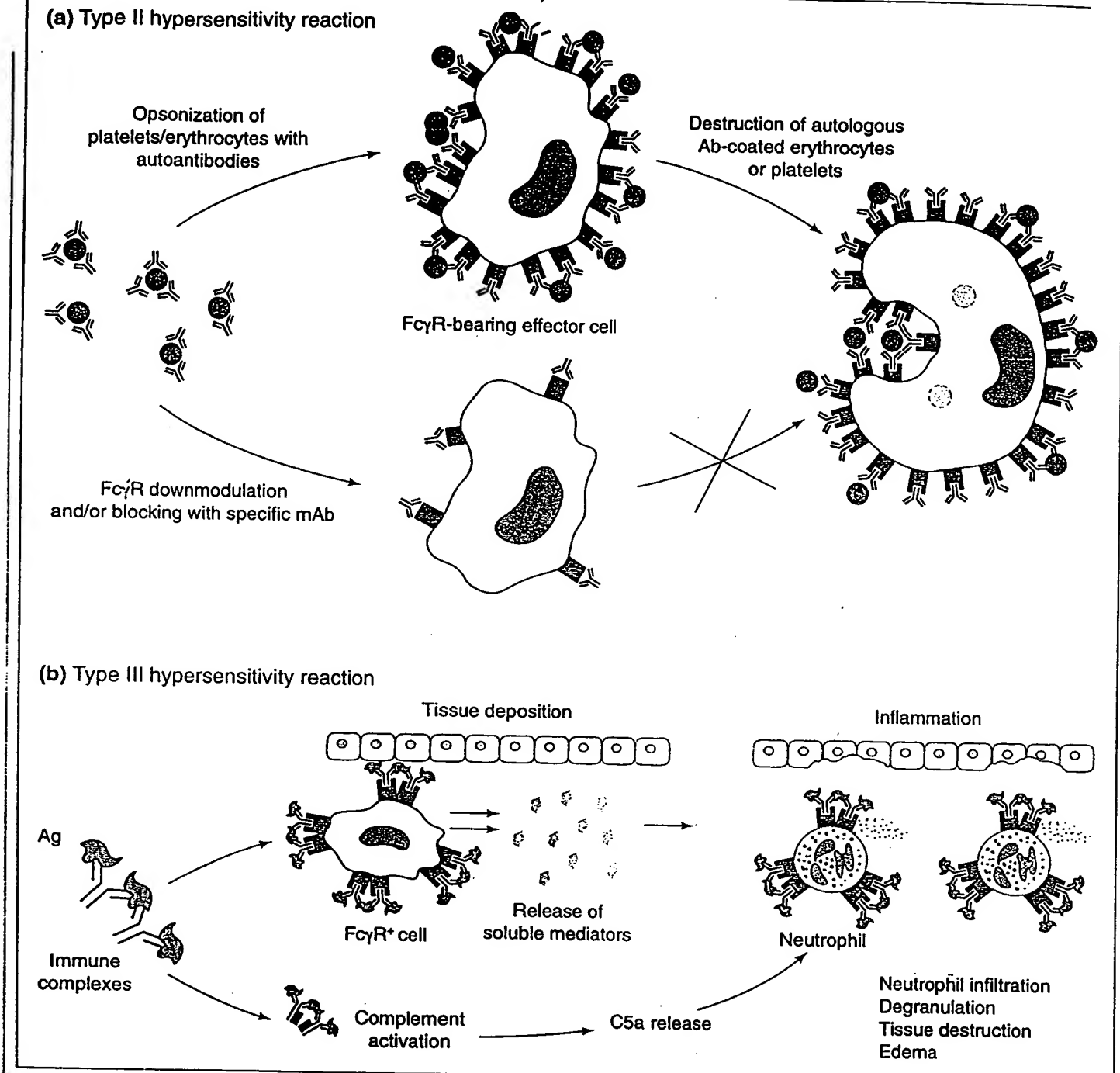


**Fig. 3.** *Role of FcγRs in type II and III hypersensitivity. (a) Type II hypersensitivity is induced when Abs bind to Ag(s) on autologous cells such as platelets or erythrocytes. Opsonized cells are then destroyed upon encountering FcγR-bearing effector cells, resulting in autoimmune disorders such as ITP (when the target cell is a platelet) or AIHA (when the target cell is an erythrocyte). ITP has been treated with molecules that downmodulate or block FcγRs to prevent platelet destruction. (b) Type III hypersensitivity is induced when circulating immune complexes that have not been properly cleared by FcγR-bearing cells of the mononuclear phagocyte system deposit at tissue sites. Ag–Ab complexes encounter FcγR-bearing cells and trigger the release of soluble mediators. These mediators initiate a series of events, including tissue edema and infiltration of neutrophils. The neutrophils mediate tissue destruction upon engagement of FcγRs by immune complexes or anti-FcγR autoantibodies. Abbreviations: Ab, antibody; Ag, antigen; AIHA, autoimmune hemolytic anemia; ITP, idiopathic thrombocytopenia purpura; mAb, monoclonal antibody.*

FcγRs are also important for immune defense to intracellular pathogens such as *Toxoplasma gondii*. Antibodies specific for *T. gondii* focus the organism to the effector cell by binding to FcγRs, thereby leading to destruction of the pathogen. BSMs that focus *T. gondii* to the surface of myeloid effectors (monocytes and neutrophils) mediate destruction of the pathogen regardless of the surface antigen on the effector cell to which they are directed[37]. In contrast to phagocytes, NK cells destroy *T. gondii* only upon targeting to FcγRIII, and not other cell-surface markers, identifying FcγRIII on NK cells as the primary trigger molecule for *T. gondii* destruction[37]. BSMs are

now being developed for a variety of microorganisms, including fungi and antibiotic-resistant bacterial strains, to target these pathogens specifically to FcγR-expressing cytotoxic effector cells.

### *Antibody-dependent enhancement*

Another interaction between pathogens and FcγRs is constituted by the phenomenon of antibody-dependent enhancement (ADE) of infection by certain viruses. Sufficient level of opsonization by virus-specific antibodies leads to FcγR crosslinking, internalization and degradation of opsonized virus particles. However, in some instances, suboptimal levels of virus-specific antibodies have been found to promote infection of FcγR$^+$ cells by flavi viruses, alpha viruses, rhabdoviruses and retroviruses[1]. Also, *in vitro*, BSMs that target dengue virus to FcγRI or FcγRIIa, or to non-FcγR surface antigens, can mediate ADE by focusing virus to the cell surface[38]. On the other hand, BSMs that direct HIV to FcγRI, FcγRII or FcγRIII on monocyte-derived macrophages markedly reduced virus production with no evidence of ADE (Ref. 39). However, a BSM targeting HIV to a non-FcγR surface antigen (CD33) was ineffective and even led to ADE of macrophages. Thus, the evidence for FcγR-mediated ADE is not conclusive.

Another gp41- and FcγRI-specific BSM (MDX-240) has been shown to decrease virus production significantly, as well as diminish formation of HIV proviral DNA in macrophages. In a phase I/II clinical trial, up to six 10 mg m$^{-2}$ doses of MDX-240 were tolerated well, and induced a transient increase in CD4$^+$ T cells in some patients, although none of the treated patients showed evidence of ADE (J.L. Pasquali *et al.*, unpublished). These studies establish the pleiotropic role of FcγRs in infectious disease processes and identify FcγR-directed BSMs as a potential therapeutic approach.

## FcRs and autoimmune disorders

FcγRs have been shown to play a significant role in autoimmune disorders, either by mediating destruction of normal cells opsonized with autoantibodies or, conversely, by failing to clear ICs adequately. For example, inability of FcγR-bearing cells to remove soluble ICs has been proposed to enhance autoimmune conditions such as systemic lupus erythematosus (SLE), where IC deposition in tissues triggers inflammation and tissue destruction, a characteristic type III hypersensitivity reaction (Fig. 3). On the other hand, engagement of functional FcRs on effector cells of the mononuclear phagocyte system triggers the destruction of autologous erythrocytes or platelets in the presence of autoantibodies directed to these cells. This may result in autoimmune hemolytic anemia (AIHA) or idiopathic thrombocytopenia purpura (ITP), both of which are autoimmune disorders characteristic of the type II hypersensitivity class of inflammation (Fig. 3). These observations suggest that FcγR-directed therapies could be developed to treat autoimmune disorders mediated by either type II or III hypersensitivity reactions.

SLE patients characteristically make autoantibodies specific for double-stranded (ds)DNA and other nuclear factors. The ICs formed by these antibodies deposit in the kidney and cause renal dysfunction because of insufficient clearance by phagocytes. FcRs in these patients may be downregulated or uncoupled from the signal transduction cascade[40,41]. FcRs may also play a role in the inflammation and tissue destruction observed in SLE patients[15]. Tissue-deposited ICs crosslink FcRs on infiltrating immune effector cells (neutrophils and macrophages), causing the release of inflammatory cytokines, proteolytic enzymes and other toxic molecules (Fig. 3)[42]. The presence of anti-FcγR autoantibodies in the sera of patients with autoimmune diseases has been proposed to explain the role of impaired FcγR function[43]. Anti-FcγRI, -II or -III autoantibodies have been purified from the sera of patients with SLE, Sjögren's syndrome, rheumatoid arthritis, Raynaud's disease and progressive systemic sclerosis. These may not only affect IC clearance, but can also crosslink FcγRs and trigger release of proinflammatory molecules[43]. Soluble FcγRs have been demonstrated to inhibit the Arthus reaction, implicating a role for FcγRs in type III hypersensitivity reaction[44]. Recent studies, demonstrating drastically reduced Arthus reaction in FcR γ-chain-deficient and FcγRIII-deficient mouse models, have established that FcγRs play an important role in type III hypersensitivity reactions[15,17].

FcγR polymorphisms also seem relevant in autoimmune disease. A marked skewing of FcγRIIa allotypes that interact differently with human IgG2 and IgG3 isotypes has been observed in Caucasian SLE patients with lupus nephritis[45], and in African–American SLE patients, both with and without lupus nephritis[46]. Several clinical parameters were found more frequently in FcγRIIa-R/R131 than in FcγRIIa-H/H131 homozygous patients, including high levels of anti-dsDNA and anti-Sm autoantibodies, as well as increased incidence of AIHA (R. Repp and J.G.J. van de Winkel, unpublished). Furthermore, this polymorphism seems important for the activation capacity of anti-neutrophil cytoplasmic antibodies in Wegener's granulomatosis[47]. Collectively, these data suggest that the FcγRIIa polymorphism constitutes a risk factor that has pathophysiological importance for IC disorders.

Recent work demonstrating the inability of anti-platelet antibodies to induce thrombocytopenia in FcR-γ-chain-deficient mice has solidified and extended the role of FcγRs in type II hypersensitivity disorders (AIHA and ITP)[16]. Corticosteroids, often the first line of treatment for ITP, have suppressive effects on FcγR functions[1], impeding the destruction of antibody-coated platelets by FcγR$^+$ cells of the mononuclear phagocyte system. Other treatments for ITP include intravenous immunoglobulin (IVIg) and anti-Rhesus factor antibody (WinRho). One proposed mechanism of action for IVIg and WinRho suggests that their binding to FcγRs on mononuclear phagocytes leads to inhibition of the Fc-mediated destruction of antibody-coated platelets[48,49]. Decreased FcR function in monocytes derived from IVIg-treated patients, and successful treatment of ITP by infusion of the Fc portion of IgG, support the idea that FcγR blockade is a relevant mechanism of action[50]. A role for FcγRs in AIHA is further supported by prolonged IC clearance in mice treated with an anti-murine FcγRII/III mAb (2.4G2)[51], and delayed clearance of antibody-opsonized erythrocytes in chimpanzees infused with an anti-FcγRIII mAb (3G8)[52]. Furthermore, an ITP patient treated with mAb 3G8 showed a dramatic, albeit transient, rise

in platelet count[53]. Treatment of an IVIg-refractory ITP patient with an anti-FcγRI mAb (197), which triggers downmodulation of FcγRI, showed significant clinical improvement[54]. Although the platelet count remained stable during the five-day mAb treatment, the patient showed a marked rise in platelets in response to subsequent IVIg treatments. A humanized anti-FcγRI mAb (H22)[55] can efficiently downmodulate FcγRI on monocytes and macrophages, resulting in inhibition of phagocytosis and ADCC of antibody-coated cells (P.K. Wallace, unpublished). Clinical trials of this reagent for evaluation of *in vivo* efficacy in ITP and AIHA patients are expected to commence soon.

## Concluding remarks

FcγRs are clinically relevant trigger molecules on both myeloid and lymphoid effector cells, and their activation and deactivation can be exploited to combat various diseases. Recently, the signal transduction pathways of FcγRs have been partially delineated and FcγR-specific mAbs and BSMs are being tested in preclinical and clinical studies with encouraging results. Novel techniques to affect directly the intracellular signaling cascade of FcγRs, and multispecific molecules that can simultaneously activate or deactivate several classes of FcγRs, may offer additional therapeutic options.

We thank T. Keler, P. Guyre and D. Drakeman for critical reading of the manuscript, and E. Clinebell and K. Patricia for their assistance in the preparation of this manuscript.

*Yashwant Deo (YashDeo@injersey.com) and Robert Graziano are at Medarex Inc., Annandale, NJ 08801, USA; Roland Repp is at the Division of Hematology/Oncology, University of Erlangen-Nürnberg, 8520 Erlangen, Germany; Jan van de Winkel is at the Dept of Immunology, University Hospital Utrecht, 3508 GA Utrecht, The Netherlands.*

## References

1 van de Winkel, J.G.J. and Capel, P.J.A. (eds) (1996) *Human IgG Fc Receptors*, R.G. Landes Company

2 Ravetch, J.V. and Kinet, J-P. (1991) *Annu. Rev. Immunol.* 9, 457–492

3 Masuda, M. and Roos, D. (1993) *J. Immunol.* 151, 7188–7195

4 Van den Herik-Oudijk, I.E., Ter Bekke, M.W.H., Tempelman, M.J., Capel, P.J.A. and van de Winkel, J.G.J. (1995) *Blood* 86, 3302–3307

5 Amigorena, S., Bonnerot, C., Drake, J.R. *et al.* (1992) *Science* 256, 1808–1812

6 Parren, P.W.H.I., Warmerdam, P.A., Boeije, L.C. *et al.* (1992) *J. Clin. Invest.* 90, 1537–1546

7 Ory, P.A., Goldstein, I.M., Kwoh, E.E., Clarkson, S.B. and Goldstein, I.M. (1989) *J. Clin. Invest.* 84, 1688–1691

8 de Haas, M., Koene, H.R., Kleijer, M. *et al.* (1996) *J. Immunol.* 156, 2948–2955

9 Salmon, J.E., Millard, S.S., Brogle, N.L. *et al.* (1995) *J. Clin. Invest.* 95, 2877–2885

10 Jones, S.L. and Brown, E.J. (1996) in *Human IgG Fc Receptors* (van de Winkel, J.G.J. and Capel, P.J.A., eds), pp. 149–163, R.G. Landes Company

11 Takai, T., Ono, M., Hikida, M., Ohmori, H. and Ravetch, J.V. (1996) *Nature* 379, 346–349

12 Wallace, P.K., Valone, F.H. and Fanger, M.W. (1995) in *Bispecific Antibodies* (Fanger, M.W., ed.), R.G. Landes Company

13 Liu, C., Goldstein, J., Graziano, R.F. *et al.* (1996) *J. Clin. Invest.* 98, 2001–2007

14 Heijnen, I.A.F.M., van Vugt, M.J., Fanger, N.A. *et al.* (1996) *J. Clin. Invest.* 97, 331–338

15 Sylvestre, D.L. and Ravetch, J.V. (1994) *Science* 265, 1095–1098

16 Clines, R. and Ravetch, J.V. (1995) *Immunity* 3, 21–26

17 Hazenbos, W.L.W., Gessner, J.E., Hofhuis, F.M.A. *et al.* (1996) *Immunity* 5, 181–188

18 Dyer, M.J.S., Hale, G., Hayhoe, F.G.J. and Waldmann, H. (1989) *Blood* 73, 1431–1439

19 Maloney, D.G., Liles, T.M., Czerwinski, D.K. *et al.* (1994) *Blood* 84, 2457–2466

20 Repp, R., Valerius, T., Sendler, A. *et al.* (1991) *Blood* 78, 885–889

21 Berends, D., van der Kwast, T.H., de Both, N.J. and Mulder, P.G. (1989) *Cancer Immunol. Immunother.* 28, 123–130

22 Adams, D.O., Hall, T., Steplewski, Z. and Koprowski, H. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 3506–3510

23 Takahashi, H., Nakada, T., Nakaki, M. and Wands, J.R. (1995) *Gastroenterology* 108, 172–182

24 Denkers, E.Y., Badger, C.C., Ledbetter, J.A. and Bernstein, I.D. (1985) *J. Immunol.* 135, 2183–2189

25 Riethmueller, G., Schneider-Gadicke, E., Schlimok, G. *et al.* (1994) *Lancet* 343, 1177–1183

26 Baselga, J., Tripathy, D., Mendelsohn, J. *et al.* (1996) *J. Clin. Oncol.* 14, 737–744

27 Elsasser, D., Valerius, T., Repp, R. *et al.* (1996) *Blood* 87, 3803–3812

28 Weiner, L.M., Holmes, M., Adams, G.P., LaCreta, F., Watts, P. and Garcia de Palazzo, I. (1993) *Cancer Res.* 53, 94–100

29 Hombach, A., Jung, W., Pohl, C. *et al.* (1993) *Int. J. Cancer* 55, 830–836

30 Valone, F.H., Kaufman, P.A., Guyre, P.M. *et al.* (1995) *J. Clin. Oncol.* 13, 2281–2292

31 Repp, R., Valerius, T., Wieland, G. *et al.* (1995) *J. Hematother.* 4, 415–421

32 Ball, E.D., Guyre, P.M., Mills, L., Fisher, J., Dinces, N.B. and Fanger, M.W. (1992) *J. Hematother.* 1, 85–94

33 Weiner, L.M., Clark, J.I., Davey, M. *et al.* (1995) *Cancer Res.* 55, 4586–4592

34 Tyler, D.S., Nastala, C.L., Stanley, S.D. *et al.* (1989) *J. Immunol.* 142, 1177–1182

35 Sanders, L.A.M., Feldman, R.G., Voorhorst-Ogink, M.M. *et al.* (1995) *Infect. Immun.* 63, 73–81

36 Salmon, J.E., Edberg, J.C., Brogle, N.L. *et al.* (1992) *J. Clin. Invest.* 89, 1276–1281

37 Erbe, D.V., Pfefferkorn, E.R. and Fanger, M.W. (1991) *J. Immunol.* 146, 3145–3151

38 Mady, B.J., Kurane, I., Erbe, D.V. *et al.* (1991) *J. Immunol.* 147, 3139–3144

39 Connor, R.I., Dinces, N., Howell, A.L., Romet-Lemonne, J.L., Pasquali, J.L. and Fanger, M.W. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 9593–9597

40 Frank, M.M., Hamburger, M.I., Lawley, T.J., Kimberly, R.P. and Plotz, P.H. (1979) *New Engl. J. Med.* 300, 518–523

41 Salmon, J.E., Kimberly, R.P., Gibofsky, A. and Fotino, M. (1984) *J. Immunol.* 133, 2525–2531

42 Boros, P., Odin, J.A., Muryoi, T., Masur, S.K., Bona, C. and Unkeless, J.C. (1991) *J. Exp. Med.* 173, 1473–1482

43 Boros, P., Muryoi, T., Spiera, H., Bona, C. and Unkeless, J.C. (1993) *J. Immunol.* 150, 2018–2024
44 Ierino, F.L., Powell, M.S., McKenzie, I.F.C. and Hogarth, P.M. (1993) *J. Exp. Med.* 178, 1617–1628
45 Duits, A.J., Bootsma, H., Derksen, R.H.W.M. *et al.* (1995) *Arthritis Rheum.* 39, 1832–1836
46 Salmon, J.E., Millard, S., Schachter, L.A. *et al.* (1996) *J. Clin. Invest.* 97, 1348–1354
47 Porges, A.J., Redecha, P.B., Kimberly, W.T., Csernok, E., Gross, W.L. and Kimberly, R.P. (1994) *J. Immunol.* 153, 1271–1280
48 Imbach, P., Barandun, S., d'Apuzzo, V. *et al.* (1981) *Lancet* i, 1228–1231
49 Bussel, J.B., Kimberly, R.P., Inman, R.D. *et al.* (1991) *Blood* 77, 1884–1893
50 Debre, M., Bonnet, M-C., Fridman, W-H. *et al.* (1993) *Lancet* 342, 945–949
51 Kurlander, R.J., Ellison, D.M., Hall, J. *et al.* (1984) *J. Immunol.* 133, 855–862
52 Clarkson, S.B., Kimberly, R.P., Valinsky, J.E. *et al.* (1986) *J. Exp. Med.* 164, 474–489
53 Clarkson, S.B., Bussel, J.B., Kimberly, R.P. *et al.* (1986) *New Engl. J. Med.* 314, 1236–1239
54 Ericson, S.G., Coleman, K.D., Wardwell, K. *et al.* (1996) *Br. J. Haematol.* 92, 718–724
55 Graziano, R.F., Tempst, P.R., White, P. *et al.* (1995) *J. Immunol.* 155, 4996–5002
56 Caron, P.C., Jurcic, J.G., Scott, A.M. *et al.* (1994) *Blood* 83, 1760–1768
57 Goldstein, J., Graziano, R.F., Sunderpandiyan, K., Somasundaram, C. and Deo, Y.M. *J. Immunol.* (in press)

0022-1767/91/1461-0018$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 146, 18–25, No. 1, January 1, 1991
Printed in U.S.A.

# POLYCLONAL ACTIVATION OF THE MURINE IMMUNE SYSTEM BY AN ANTIBODY TO IgD

## XI. Contribution of Membrane IgD Cross-Linking to the Generation of an in Vivo Polyclonal Antibody Response[1]

DIANA K. GOROFF,* JOANNE M. HOLMES,* HERVE BAZIN,† FRANCOISE NISOL,† AND FRED D. FINKELMAN[2*]

*From the *Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799; and †Experimental Immunology Unit, Faculty of Medicine, University of Louvain, B-1200 Brussels, Belgium*

**The injection of mice with a foreign, polyclonal antibody to IgD sequentially induces: 1) activation of B cells by cross-linking of their cell membrane (m) IgD; 2) B cell processing and presentation of the bound anti-IgD antibody to T cells; 3) activation of these T cells; and 4) T-dependent stimulation of B cell differentiation into IgG1 secreting cells. To determine whether the cross-linking of B cell membrane IgD and/or the resulting B cell activation that follows contribute to the generation of the polyclonal IgG1 response, we examined the abilities of three sets of anti-δ mAb or mAb fragments to stimulate polyclonal IgG1 production. Within each set mAb were matched for species and Ig isotypic determinants, but differed in avidity for IgD or in ability to cross-link IgD. In addition, experiments were performed to determine whether the anti-δ mAb had to be foreign to the immunized mouse to stimulate an IgG1 response. Results of these experiments indicate that: 1) recognition of the injected anti-δ antibody as foreign is required for the induction of a polyclonal IgG1 response; 2) the cross-linking of B cell membrane Ig, which directly activates B cells, can contribute considerably to the generation of in vivo IgG1 production; and 3) that even relatively weak cross-linking of membrane Ig by ligands that bind it with low avidity can make this contribution.**

The cross-linking of B cell m[3]Ig has been shown both in vitro and in vivo to activate B lymphocytes (1–6) and to make them more responsive to cytokines that can stimulate antibody production (7–10). Although much progress has been made in the past few years toward understanding the cellular physiology of mIg cross-linking-mediated B cell activation (11–20), the role of this process in the generation of a humoral immune response is largely undefined. mIg cross-linking most likely contributes to the generation of in vivo antibody responses to "type 2" T cell-independent Ag. These Ag have multiple representations of the same epitope that enhance their ability to crosslink the mIg of Ag-specific B cells (21, 22). Stimuli that derive from mIg cross-linking by these Ag are the sole mechanism by which they appear to be able to stimulate B cells in an Ag-specific manner, since they lack the polyclonal activating properties of bacterial LPS and are unable to induce T cell help (23, 24).

Little is known, however, about the importance of mIg cross-linking for the in vivo generation of antibody responses to T cell-dependent Ag. Although some in vitro studies of T cell-dependent B cell activation suggest that mIg cross-linking may act synergistically with T cell-dependent stimuli to induce B cell activation (7, 8, 25, 26), other studies suggest that any contribution made by mIg cross-linking to T cell-induced B cell activation is minor (27). In addition, while it is generally agreed that B cell mIg plays a critical role in B cell processing and presentation of Ag to T cells (27–35), the role of mIg cross-linking in the processing and/or presentation of mIg-bound Ag remains controversial. Although activated B cells have been reported to be better than small resting B cells at presenting Ag that they have bound through surface receptors other than mIg to T cells (36, 37), univalent Fab fragments of anti-Ig antibodies, which neither cross-link mIg nor activate B cells, have been found to be as efficacious at enhancing Ag presentation by resting B cells as the B cell-activating, divalent F(ab')2 fragments of the same antibodies (27, 34, 38–40). The ability of soluble protein Ag that have only a single representation of any epitope to stimulate an in vivo antibody response suggests that mIg cross-linking may not be essential. Such Ag, however, are generally only strongly immunogenic if polymerized or administered with adjuvants that might, such as alum, polymerize them directly through surface adsorption, or such as CFA, enhance their uptake by an APC (41). Furthermore

Received for publication July 2, 1990.
Accepted for publication October 9, 1990.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] This work was supported in part by National Institutes of Health Grant RO1 AI21328, Uniformed Services University of the Health Sciences Protocol RO 8308, Office of Naval Research and Development Command Grant N00007590WR00008, and the Fonds National de la Recherche Scientifique (Belgium). The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The research reported herein was conducted according to the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, HHS Pub. No. (NIH) 85-23, revised 1985.
[2] Address correspondence and requests for reprints to Dr. Fred D. Finkelman, Department of Medicine, Room A3060, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.
[3] Abbreviations used in this paper: m, cell membrane; GaMδ, affinity purified goat antibody specific for mouse IgD.

even if mIg cross-linking is not essential to the induction of a T cell-dependent antibody response, it might still enhance such a response.

To investigate the role of mIg cross-linking in in vivo T cell-dependent antibody production we have used a system in which the injection of mice with anti-IgD antibodies stimulates a large, polyclonal IgG1 response 7 to 9 days after injection (6, 42). Studies in this system revealed that injection of GaMδ first cross-links the mIgD on B lymphocytes, which stimulates T cell-independent increases in B cell size, expression of MHC class II and other activation-related Ag, and DNA synthesis (6, 43). GaMδ is internallized by these B cells and is presented to goat IgG-specific T cells, with the result that polyclonal T cell activation is observed starting 2 to 3 days after GaMδ injection (6, 44, 45). This T cell activation is essential for the production of both the polyclonal and the goat IgG-specific IgG1 responses that start 3 days later (42). More recently we have investigated the abilities of different anti-δ mAb, and fragments of these antibodies to cross-link mIgD and to activate B cells. These studies have demonstrated that those antibodies that have the greatest ability to cross-link IgD also have the greatest ability to activate B cells in vitro and in vivo, whereas those antibodies that have more limited ability to cross-link IgD have little ability to stimulate B cell DNA synthesis, but can still induce B cells to increase their expression of class II MHC (46). We now report studies in which the abilities of these, and additional anti-δ mAb to induce in vivo polyclonal IgG1 responses are investigated. Our experiments indicate that although mIg cross-linking can contribute considerably to the generation of an in vivo IgG1 response, the extent of mIg cross-linking required for this contribution is small, and can be achieved with even low affinity anti-δ antibodies that are unable to induce B cell DNA synthesis.

## MATERIALS AND METHODS

*Mice.* BALB/c female mice were obtained from the Small Animals Division of the National Cancer Institute, National Institutes of Health, Bethesda, MD, and were used at 8 to 12 wk of age. CB20 mice, which are congenic to BALB/c mice but express Ig of the *b* allotype, were a gift of Dr. Michael Potter (National Cancer Institute). (BALB/c × CB20)F1 mice were bred in the animal facility of the Department of Laboratory Animal Medicine, USUHS.

*Antibodies.* The production, preparation, purification, and characterization of the following antibodies and antibody fragments have been described previously: Hδ$^a$/1 is a mouse IgG2b of the *b* allotype that binds with high affinity to a determinant on the Fc fragment of IgD of the *a* allotype (47). This antibody is an effective crosslinker of both soluble and cell membrane IgD, and induces B cells from *a* allotype mice to increase their expression of MHC class II, enlarge in size, and synthesize DNA (46, 47). A monovalent Fab/Fc fragment of this antibody, which is composed of a single Fab fragment linked to the Fc fragment by the hinge region (48), was produced by elastase digestion and purified by protein A affinity chromatography and Sephadex G-150 gel filtration (49). The purified Fab/Fc fragment preparation used contained less than 2% intact Hδ$^a$/1. This fragment, unlike Fab, has a long in vivo half-life. It is capable of activating B cells in vivo by FcγRII-dependent mechanism, which is blocked by a rat IgG2b mAb to this receptor, 24G2 (50). AMS-15, AF4.70, and FF1-4D5, are all IgG2a antibodies of the *b* allotype that bind antigenic determinants associated with the Fab or F(ab')2 fragments of IgD of the *a* allotype (46, 51). AMS-15 and FF1-4D5 both bind these determinants with relatively high avidity, whereas AF4.70 binds with very low affinity (46). None of these antibodies cross-link IgD well or directly induce B cells to synthesize DNA; however, they have some cross-linking ability and are able to induce B cells to increase MHC class II expression (46). Affinity purified, subclass-specific, rabbit anti-mouse IgG1 antibody and mouse IgG-absorbed goat anti-rabbit Ig antibody (a gift of Dr. Ellen Vitetta, Dallas, TX) were prepared as described (42). Three rat IgG2a mAb to mouse IgD were used. 11-26, was a gift of Dr. John Kearney (University of Alabama School of Medicine, Birmingham, AL), while HBδ-6 (also known as LO-MD-6) and HBδ-7 (also known as LO-MD-7) (52) were prepared in the laboratory of Dr. Herve Bazin (University of Louvain Faculty of Medicine, Brussels, Belgium). All were grown as ascites in LOU/C Igκ1b (OKA) rats, and were purified by sequential $(NH_4)_2SO_4$ precipitation and DE-52 (Whatman Inc., Clifton, NJ) ion exchange chromatography, as previously described (46). Characterization of these mAb is described in *Results*. B3B4, a rat IgG2a mAb specific for the mouse low affinity FcεRII (CD23) (53) was a gift of Dr. Daniel Conrad, Richmond, VA. MKD6, an anti-I-A$^d$ mAb (54), was a gift of Dr. James Mond, Bethesda, MD.

*Quantitation of serum IgG1.* Serum IgG1 content was analyzed by radial immunodiffusion. Radial immunodiffusion plates were either purchased from Meloy or produced with rabbit anti-mouse IgG1 antibody as described (55). An IgG1 standard was purchased from Meloy Laboratories, Inc., Springfield, VA.

*Immunofluorescence studies.* Immunofluorescence microscopy with a Leitz Ortholux II microscope was used to study the abilities of FITC-labeled anti-δ mAb to cap B cell mIgD as previously described. Flow microfluorimetry with a Becton Dickinson FACS II or a Becton Dickinson FACScan (Mountain View, CA) was used to quantitate MHC class II (Ia) expression on spleen cells stained with a FITC-labeled anti-I-A$^d$ mAb (MKD6), for measurements of anti-δ mAb avidity, and for determination of the fine specificity of rat anti-δ mAbs, as described (6, 46).

## RESULTS

*Characterization of rat IgG2a anti-mouse IgD mAb.* Three rat IgG2a anti-mouse δ mAb, 11-26, HBδ-6, and HBδ-7, were characterized for their fine specificities, avidities, cross-linking abilities, and abilities to induce increases in splenic B cell class II MHC expression in vivo and DNA synthesis in vitro. To determine whether these mAb bound to δFc or δFd determinants, spleen cells from BALB/c mice, which express Ig of the *a* allotype, and spleen cells from *b* allotype-congenic CB20 mice were preincubated on ice with unlabeled Hδ$^a$/1, which binds to a determinant on the Fc part of IgD of the *a* allotype but not to IgD of the *b* allotype (47), or with a control mAb (CBPC-101). Cells were then washed, stained on ice with FITC-labeled 11-26, HBδ-6, or HBδ-7, and analyzed for fluorescence intensity by flow microfluorimetry. Although all three FITC-labeled mAb brightly stained approximately 50% of both BALB/c and CB20 spleen cells, pretreatment with unlabeled Hδ$^a$/1 almost completely inhibited staining of BALB/c spleen cells by HBδ-6 and HBδ-7, but did not affect staining of these cells by 11-26, and, as expected, had no effect on staining of CB20 spleen cells by any of the antibodies tested (data not shown). Thus, HBδ-6 and HBδ-7 are specific for a determinant or determinants on δFc. To determine if 11-26 binds to a δFd-related determinant, we examined the ability of two mAb that are specific for determinants on δFd of the *a* allotype, AMS-15 and FF1-4D5 (46) to inhibit staining of BALB/c B cells by FITC-11-26. Surprisingly, neither antibody blocked staining (data not shown). However, when analyzed in an ELISA, 11-26 bound well to TEPC-1017, a monoclonal IgD that contains both δFd and δFc determinants, but failed to bind to a monoclonal IgD, KWD-1, that lacks δFd ($C\delta_1$) determinants (56), whereas Hδ$^a$/1 bound equally well to both TEPC-1017 and KWD-1 (data not shown). Thus, 11-26 is specific for a δFd-related determinant, but one different from those bound by FF1-4D5 and AMS-15.

To compare the avidities of 11-26, HBδ-6, and HBδ-7, we determined both the concentrations of these mAb required to half-maximally stain splenic B cells ($MFI_{50}$), as well as the concentration of a purified IgD plasmacy-

toma protein (TEPC-1017) required to inhibit staining by $MFI_{50}$ concentrations of these mAb by an additional 50% (i.e., to reduce staining to 25% of the maximum level) ($IC_{50}$). Intensity of staining was determined by flow microfluorimetry. A high avidity antibody will stain cells maximally at a low concentration and will be neutralized by a low concentration of IgD, and hence, will have a low $MFI_{50}$ and $IC_{50}$. All three mAb had similar $MFI_{50}$ and $IC_{50}$ values (Table I) which were comparable to those of previously characterized high avidity allo-anti-δ mAb. Thus, all three mAb bind IgD with high avidity.

Our previous studies of allo-anti-δ mAb revealed that all those specific for δFc could effectively cross-link both soluble and cell membrane-bound IgD, whereas those specific for δFd had relatively little cross-linking ability (46). The rat anti-δ mAb followed this trend. Both HBδ-6 and HBδ-7 precipitated TEPC-1017 as tested by gel double diffusion, whereas 11-26 did not. Similarly, B cell mIgD was capped within 15 min by HBδ-6 and HBδ-7, but not by 11-26 (data not shown).

The abilities of these mAb to activate B cells were studied in vivo and in vitro. Injection of BALB/c mice with 100 μg of HBδ-6 or HBδ-7 stimulated greater than 100% increases in $Ia^d$ expression one day later, as determined by flow microfluorimetric analysis. Injection of an equal quantity of 11-26, however, stimulated only a 13% increase in the fluorescence intensity of cells stained with anti-$Ia^d$ antibody (Table II). This low degree of stimulation, while reproducible in several experiments, was considerably less than that seen even with those allo-anti-δ mAb that have a limited ability to cross-link mIgD

TABLE I

*Avidities of rat IgG2a anti-mouse δ mAb*[a]

| Antibody | $MFI_{50}$ | $IC_{50}$ |
|---|---|---|
| 11-26 | 1.6 | 1.5 |
| HBδ-6 | 3.0 | 1.0 |
| HBδ-7 | 2.0 | 0.8 |

[a] To determine that concentration of each anti-mouse δ mAb required to bind 50% of B cell IgD molecules ($MFI_{50}$), BALB/c spleen cells were exposed for 30 min at 0°C to 0.1 to 100 μg/ml of 11-26, HBδ-6, or HBδ-7, washed, sandwich stained with FITC-labeled rabbit anti-mouse IgG2 antibody, rewashed, and analyzed for surface fluorescence by flow microfluorimetry. The $MFI_{50}$ is that concentration of anti-δ mAb that stained $mIgD^+$ cells to half-maximal intensity. To determine that concentration of IgD required to inhibit staining of $mIgD^+$ cells by the $MFI_{50}$ concentration of anti-δ mAb by 50% ($IC_{50}$), the $MFI_{50}$ concentrations of the mAb shown were preincubated with 0.1 to 100 μg of purified mouse IgD for 30 min, after which spleen cells were incubated on ice with the mixture for 30 min, washed, sandwich stained with FITC-$RaM_{\gamma}2$ antibody, and analyzed for surface fluorescence by flow microfluorimetry. $MFI_{50}$ and $IC_{50}$ values are shown in μg/ml. High avidity antibodies have low $MFI_{50}$ and high $IC_{50}$ values.

TABLE II

*Effect of in vivo treatment of mice with rat IgG2a anti-mouse δ mAb on B cell class II MHC expression*[a]

| Antibody injected | Median Fluorescence Intensity of $Ia^{d+}$ Cells |
|---|---|
| None | 161 |
| Rat IgG2a | 164 |
| 11-26 | 185 |
| HBδ-6 | 400 |
| HBδ-7 | 375 |

[a] BALB/c mice (three/group) were left untreated or were each injected i.v. with 100 μg of a control rat IgG2a mAb or with 100 μg of the anti-δ mAb shown. Mice were killed 24 h later, and their spleen cells were stained with FITC-labeled anti-$Ia^d$ mAb (MKD6) and analyzed for surface fluorescence by flow microfluorimetry. The median fluorescence intensity (average brightness) of $Ia^{d+}$ cells was determined individually for spleen cells from each mouse; mean values of the three determinations made for each group are shown.

TABLE III

*Effect of in vivo treatment of mice with allo-anti-IgD mAb on B cell class II MHC expression*[a]

| Antibody injected | No. of Expt. | Percent increase in $Ia^d$ MFI |
|---|---|---|
| Hδ°/1 | 6 | 170 ± 7 |
| AMS-15 | 3 | 76 ± 9 |
| FF1-4D5 | 3 | 77 ± 3 |
| AF4.70 | 5 | 89 ± 12 |

[a] In several independent experiments, BALB/c mice (three/group in each experiment) were left untreated or were injected i.v. with 100 μg of the anti-δ mAb shown. Mice were killed 24 h later and their spleen cells were stained with FITC-labeled anti-$Ia^d$ mAb (MKD6) and analyzed for surface fluorescence by flow microfluorimetry. Values for median fluorescence intensity (MFI) of spleen cells positively stained with anti-$Ia^d$ mAb were compared between untreated and anti-δ mAb-treated mice in each experiment. Percent increases in Ia staining of spleen cells from mice treated with each of the anti-δ mAb were calculated and results obtained in different experiments were averaged. Arithmetic means and SE are shown.

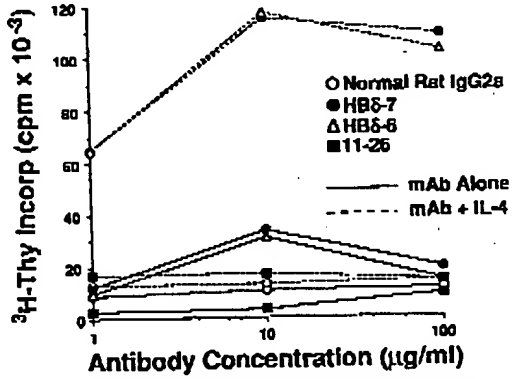


*Figure 1.* Induction of B cell DNA synthesis by rat IgG2a anti-mouse IgD mAb. T cell-depleted BALB/c spleen cells were cultured for three days in 96-well microtiter plates at $2 \times 10^5$ cells in 100 μl of culture medium with 1 to 100 μg of normal rat IgG2a, HBδ-6, HBδ-7, or 11-26, ± 100 U/ml of rIL-4. Wells were pulsed with 1 μCi of $^3$H-thymidine 18 h before cells were harvested onto glass fiber discs and discs were evaluated for radioactivity by scintillation spectroscopy. Cultures were performed in triplicate; mean values are shown.

(46) (Table III), and suggests that the mIgD-cross-linking ability of 11-26 may be extremely limited.

To determine the abilities of the three rat anti-δ mAb to stimulate B cell DNA synthesis in vitro, purified B cells were cultured for 3 days with 1 to 100 μg of each of these antibodies or a control antibody in the presence or absence of 100 U of mouse rIL-4 (a gift of Dr. Alan Levine, St. Louis, MO), then pulsed with $^3$H-thymidine, harvested, and analyzed for $^3$H content by scintillation spectroscopy (Fig. 1). Both HBδ-6 and HBδ-7 induced significant increases in DNA synthesis in the absence of IL-4, and much larger increases in the presence of this cytokine, whereas 11-26 failed to induce increases in DNA synthesis in the absence or presence of IL-4. Thus, as observed previously with allo-anti-mouse δ mAb, the ability to cross-link IgD correlated well with the ability to stimulate B cell DNA synthesis in vitro (46).

*Determination of abilities of rat IgG2a anti-mouse δ mAb to stimulate an in vivo, polyclonal IgG1 response.* To determine the abilities of the rat anti-mouse δ mAb that we had characterized to induce a polyclonal IgG1 response when injected into BALB/c mice, mice were injected with 20 to 640 μg of 11-26, HBδ-6, or HBδ-7, in the presence or absence of 500 μg of 24G2 (rat IgG2b anti-mouse $Fc\gamma RII$), and were bled 9 days later. HBδ-7 stimulated a large increase in serum IgG1 level (Fig. 2) at a dose of 40 μg/mouse, whereas the response to HBδ-

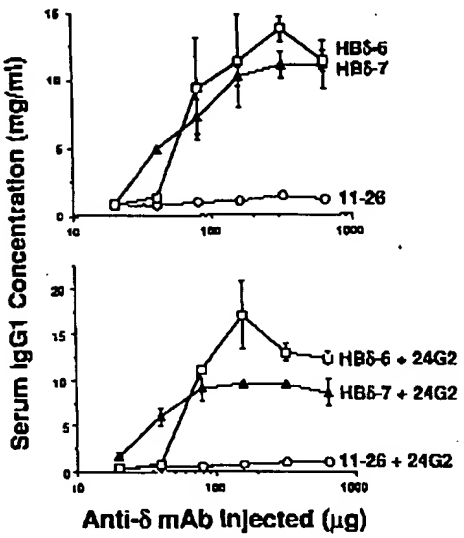


*Figure 2.* Stimulation of polyclonal IgG1 responses by rat IgG2a anti-mouse IgD mAb. BALB/c mice (five/group) were injected i.v. with 20 to 640 μg of purified 11-26, HBδ-6, or HBδ-7 (*upper panel*), or with the same antibodies plus 500 μg of 24G2 (*lower panel*). Mice were bled 9 days after mAb injection and sera were analyzed for IgG1 content by radial immunodiffusion. Geometric means and SE are shown.

was first seen at a dose of 80 μg/mouse. Responses to both antibodies reached peak or plateau levels at a dose of 160 to 320 μg/mouse. In contrast, 11-26 failed to induce more than a very small IgG1 response at any dose tested. Repeat bleeds made 13 days after 11-26 injection showed no further increases in serum IgG1 level (not shown). Injection of 24G2 by itself had no effect on serum IgG1 levels, and this antibody failed to substantially modify the responses made to any of the rat anti-mouse δ mAb (Fig. 2). Inasmuch as HBδ-6 and HBδ-7 effectively cross-link IgD and activate B lymphocytes directly, whereas 11-26, which binds well to IgD, fails to have these effects, these observations suggest that mIgD cross-linking capacity is an important determinant of the ability of an anti-δ antibody to stimulate a polyclonal IgG1 response in vivo.

*Stimulation of in vivo polyclonal IgG1 response by intact, divalent, alloanti-δ mAb that effectively cross-links mIgD and by its monovalent Fab/Fc fragment.* We have previously shown that Hδ$^a$/1, a monoclonal IgG2b of the *b* allotype that binds to and effectively crosslinks IgD of the *a* allotype, is an effective stimulator of B cell activation both in vitro and in vivo. The univalent Fab/Fc fragment of this antibody has some ability to activate B cells when injected in vivo. This in vivo B cell-activating ability appears to depend on an interaction between Hδ$^a$/1 Fab/Fc with FcγRII receptor-bearing cells, which may indirectly crosslink the mIgD to which the Hδ$^a$/1 Fab/Fc has bound. The ability of a monoclonal anti-FcγRII antibody, 24G2, to completely block Hδ$^a$/1 Fab/Fc induction of increased B cell Ia expression in vivo, without affecting the ability of intact Hδ$^a$/1 to induce increased Ia expression (49), supports this view. Inasmuch as Fab/Fc fragments, unlike univalent Fab fragments, have a long in vivo half-life and possess all of the antigenic determinants of intact IgG, comparison of the abilities of intact Hδ$^a$/1 and Hδ$^a$/1 Fab/Fc, in the presence or absence of 24G2, provides a well controlled way to study the possible contribution of mIg cross-linking to the generation of a T-dependent antibody response. Injection of BALB/c mice with 100 μg of either intact Hδ$^a$/1 or Hδ$^a$/1 Fab/Fc stimulated a considerable increase in serum IgG1 9 days later. However, 24G2 almost completely inhibited the ability of this dose of Hδ$^a$/1 Fab/Fc to induce an IgG1 response, whereas it considerably enhanced the ability of intact Hδ$^a$/1 to do so (Fig. 3). These effects of 24G2 were not shared by another rat IgG2 mAb, B3B4, which binds to CD23 (FcεRII) (53). This mAb, which fails to inhibit the ability of Hδ$^a$/1 Fab/Fc to activate B cells in vivo (49), also has little effect on the ability of either intact Hδ$^a$/1 or Hδ$^a$/1 Fab/Fc to induce polyclonal IgG1 production. These observations indicate that an interaction between FcγRII on one cell and Hδ$^a$/1 Fab/Fc that has bound to mIgD on a second cell, which promotes cross-linking of the Hδ$^a$/1-Fab/Fc-bound mIgD, contributes greatly to the generation of a polyclonal IgG1 response in mice injected with this mAb fragment. The mechanism by which anti-FcγRII mAb enhances the IgG1 response to intact Hδ$^a$/1 is less clear, and could result either from blocking of an interaction between FcγRII and mIgD on the same cell, which could inhibit B cell activation and differentiation (57, 58), or from T cell recognition of the additional foreign determinants on the rat IgG2b anti-FcγRII antibody molecule.

To determine if increasing the dose of Hδ$^a$/1 Fab/Fc injected with 24G2 would allow a large polyclonal IgG1 response to be induced, a dose-response study was performed (Fig. 4). In the presence of 24G2, a considerable increase in serum IgG1 level was induced by as little as 40 μg of intact Hδ$^a$/1, although little IgG1 production was stimulated by less than 320 μg of Hδ$^a$/1 Fab/Fc; 40 μg of intact Hδ$^a$/1 induced a larger IgG1 response than did 640 μg of Hδ$^a$/1 Fab/Fc; and 640 μg of intact Hδ$^a$/1 stimulated an IgG1 response that was more than 14-fold larger than that induced by Hδ$^a$/1 Fab/Fc. However, unlike 11-26, a high dose of Hδ$^a$/1 Fab/Fc in the presence of 24G2 can stimulate a several-fold increase in IgG1 production.

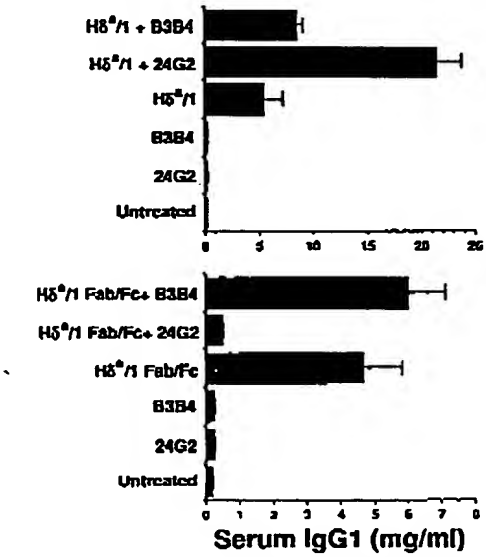


*Figure 3.* Anti-FcγRII mAb has opposite effects on the stimulation of a polyclonal IgG1 response by monovalent and divalent forms of an IgG2b anti-IgD mAb. BALB/c mice (five/group) were left untreated or were injected i.v. with 500 μg of B3B4 anti-FcγRII mAb or 24G2 anti-FcγRII mAb, or with 100 μg of intact Hδ$^a$/1 (*upper panel*) or Hδ$^a$/1 Fab/Fc (*lower panel*), or with combinations of anti-FcR and anti-δ mAb as indicated. Mice were bled 9 days after antibody injection and sera were analyzed for IgG1 content by radial immunodiffusion. Geometric means and SE are shown.

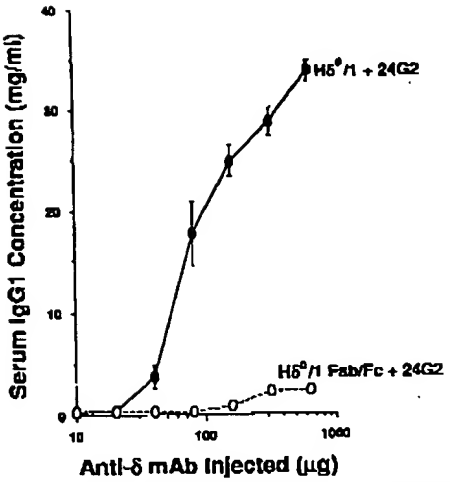


*Figure 4.* Stimulation of polyvalent IgG1 antibody production by monovalent and divalent forms of an allo-anti-δ mAb. BALB/c mice (three/group) were injected i.v. with 500 μg of 24G2 plus 10 to 640 μg of either intact Hδ^a/1 or Hδ^a/1 Fab/Fc. Mice were bled 9 days after mAb injection and serum IgG1 content was determined by radial immunodiffusion. Geometric means and SE are shown.

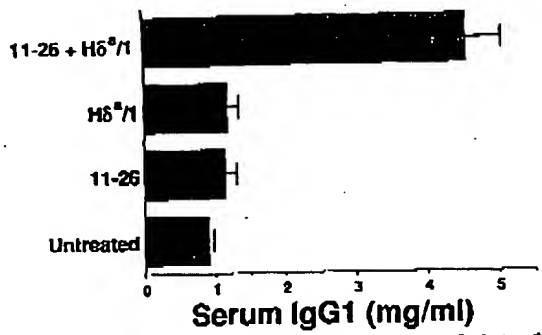


*Figure 5.* Synergy between a foreign anti-δ mAb that fails to effectively cross-link mIgD and a self mAb that effectively cross-links mIgD in the induction of a polyclonal IgG1 response. (BALB/c × CB20)F1 mice (five/group), which are heterozygous for the Ig *a* and *b* allotypes, were left untreated or were injected i.v. with 160 μg of 11-26 (rat IgG2a anti-δ mAb that does not effectively cross-link mIgD), 100 μg of Hδ^a/1 (mouse IgG2b of the *b* allotype anti-IgD of the *a* allotype, that effectively cross-links mIgD of the *a* allotype) or with both of these mAb, and were bled 9 days later. Serum IgG1 levels were determined by radial immunodiffusion. Geometric means and SE are shown.

*Synergistic stimulation of polyclonal IgG1 response in (a × b allotype)F1 mice by an anti-δ mAb that effectively cross-links mIgD but is not recognized as foreign and anti-δ mAb that fails to effectively cross-link mIgD but is recognized as foreign.* IgG2 mAb of the *b* allotype that bind to IgD of the *a* allotype fail to stimulate a polyclonal IgG1 response when injected into (*a* × *b* allotype)F1 mice, because these mice, which have circulating IgG antibodies of both the *a* and the *b* allotype, lack T cells that recognize these allo-anti-δ mAb as foreign. The injection of such mice with Hδ^a/1, for example, selectively cross-links the mIgD on the 50% of mIgD^a B cells in these mice that express IgD of the *a* allotype and activates these cells, but fails to activate T cells of these mice or to stimulate polyclonal IgG1 production (S. Morris, manuscript in preparation). We determined if a combination of Hδ^a/1, which would effectively cross-link mIgD but not be recognized as foreign by these mice, and the rat IgG2a anti-δ mAb, 11-26, which fails to effectively cross-link mIgD but is foreign to mice, would synergistically stimulate a polyclonal IgG1 response by such mice. Inasmuch as 11-26 and Hδ^a/1 bind to different parts of the IgD molecule and do not inhibit each other's binding, Hδ^a/1-induced capping and endocytosis of mIgD would also cause the internalization of 11-26 that had bound to mIgD. F1 progeny of BALB/c (*a* allotype) mice and CB20 mice, which are congenic to BALB/c mice but express Ig of the *b* allotype, were used for this experiment. Injection of these mice with either 100 μg of Hδ^a/1 or 160 μg of 11-26 failed to induce a significant increase in serum IgG1 level, whereas injection of both mAb induced a four- to five-fold increase (Fig. 5). Injection of (*a* × *b* allotype)F1 mice with a combination of Hδ^a/1 and either normal rat IgG or rat IgG2b anti-FcγRII (24C2) failed to induce a polyclonal IgG1 response (data not shown). These observations, thus, provide further evidence that mIg cross-linking, whereas not sufficient to induce an in vivo polyclonal Ig response, can contribute to such a response.

*Comparison of abilities of mouse IgG2a mAb that differ in avidity for IgD to stimulate in vivo polyclonal IgG1 response.* Inasmuch as the univalent Fab/Fc fragment of Hδ^a/1 would be expected to bind to IgD less avidly than intact Hδ^a/1, it was possible that the decreased avidity of this fragment for IgD, rather than its decreased cross-linking ability, was responsible for its diminished ability to induce an IgG1 response in vivo in the presence of anti-FcγRII. To examine this possibility, and to test the relationship between avidity of anti-δ mAb and their ability to stimulate an in vivo polyclonal IgG1 response, BALB/c mice were injected with 20 to 640 μg of one of three mouse IgG2a anti-δ mAb that differed markedly in avidity and their serum IgG1 levels were measured. The three mAb tested, AMS-15, AF4.70, and FF1-4D5, all bind IgDFd of the *a* allotype. AF4.70 has such low avidity for IgD of the *a* allotype that it stains B cells that express this allotype poorly, and this weak staining of B cells by FITC-AF4.70 is difficult to inhibit by even high concentrations of soluble IgD (46). In addition, AF4.70 exhibits barely detectable binding to ELISA wells coated with even high concentrations of soluble IgD (Fig. 6, *main graph*). FF1-4D5 stains mIgD^+ B cells well, and binds with moderately high avidity to soluble IgD. In an ELISA in which high avidity antibodies are characterized by their abilities to bind to wells coated with low Ag concentrations (59), FF1-4D5, however, appeared to have a lower avidity for IgD than either Hδ^a/1 or its Fab/Fc fragment (Fig. 6, *main graph*). The apparently more avid binding of IgD by intact Hδ^a/1 and its Fab/Fc fragment, as compared to its binding by FF1-4D5 and AF4.70, is not a result of better binding of the rabbit anti-mouse IgG2 antibody used in this ELISA to IgG2b antibodies than to IgG2a antibodies. This is demonstrated by the observation that our rabbit anti-mouse IgG2 antibody bound equivalently to Hδ^a/1 and FF1-4D5 when both anti-δ mAb were applied directly to microtiter plate wells (Fig. 6, *insert*). In other assays, AMS-15 has been found to bind soluble and mIgD at least as avidly as Hδ^a/1 (60).

None of the IgG2a alloanti-δFd antibodies is a strong cross-linker of B cell mIgD, and none is able to induce B cells to proliferate in vitro. However, all have some cross-linking and B cell activating ability, and surprisingly despite their marked differences in avidity, all have similar abilities to induce B cells to increase MHC class II expression in vivo (Table III). All of these antibodies also were able to induce a large polyclonal IgG1 response whe

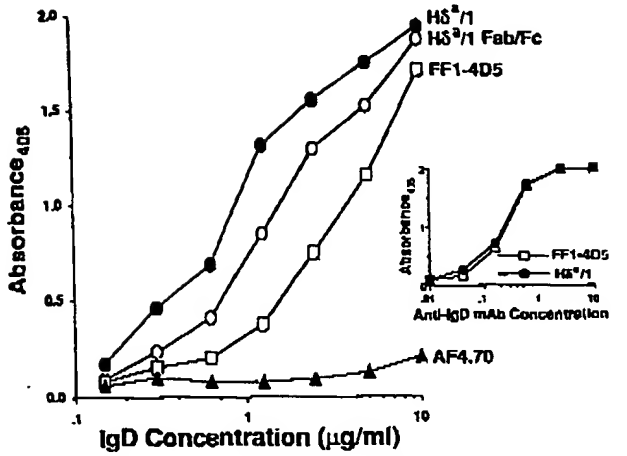


*Figure 6.* Avidity of mouse allo-anti-δ mAb. *Main graph.* Round-bottomed polyvinyl microtiter plate wells were coated with 0.15 to 10 μg/ml of a purified mouse IgD plasmacytoma protein and blocked with OVA. A total of 50 μl of 10 μg/ml of Hδ^a/1, Hδ^a/1 Fab/Fc, FF1-4D5, or AF4.70 were then added to coated wells, to which, after washing, rabbit anti-mouse γ2 antibody, alkaline phosphatase-labeled goat anti-rabbit Ig, and substrate were added sequentially. $A_{405}$ was determined for each well with an automated microtiter plate reader. Duplicate values were obtained for each mAb at each IgD concentration; mean values are shown. *Inset graph.* Round bottomed polyvinyl microtiter plate wells were coated directly with 0.01 to 10 μg of either Hδ^a/1 or FF1-4D5 and blocked with OVA. Rabbit anti-mouse γ2 antibody, alkaline phosphatase-labeled goat anti-rabbit Ig, and substrate were added sequentially. $A_{405}$ values were determined in duplicate as above.

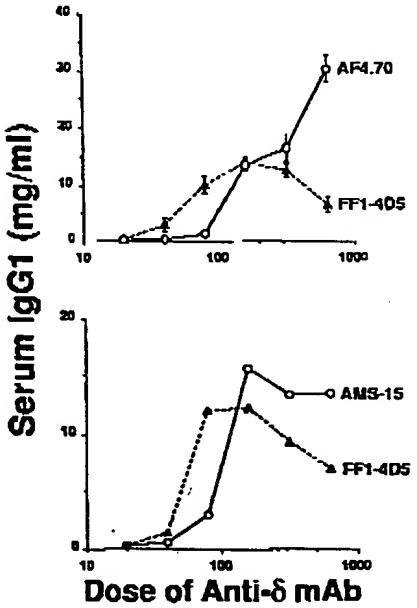


*Figure 7.* Determination of quantities of IgG2a allo-anti-mouse δ mAb required to induce a polyclonal IgG1 response in BALB/c mice. BALB/c mice (five/group) were injected i.v. with 20 to 640 μg of AF4.70 or FF1-4D5 (*upper panel*) or with AMS-15 or FF1-4D5 (*lower panel*). Mice were bled 9 days after mAb injection and serum IgG1 concentrations were determined by radial immunodiffusion. Geometric means and SE are shown.

injected into BALB/c mice (Fig. 7). FF1-4D5 was able to induce this response at approximately half the dose of either AMS-15 or AF4.70. Anti-FcγRII mAb, injected simultaneously with any of these anti-δ mAb, had little effect on their abilities to induce polyclonal IgG1 production (data not shown), possibly because it fails to block their binding to FcγRI, the high affinity, IgG2a-specific FcR that is expressed by macrophages (61). Supplementation of any of these IgG2a alloanti-δ mAb with CBPC-101, a mouse IgG2a of the *b* allotype that does not bind to any murine Ag, also had no effect on the abilities of these mAb to stimulate a polyclonal IgG1 response (data not shown). Thus, even low avidity binding of an anti-δ mAb to mIgD, as long as it has some ability to cross-link mIgD and activate B cells, is able to induce a T-dependent polyclonal IgG1 response.

## DISCUSSION

The experiments described in this report were performed to determine whether the ability of an antigen to cross-link mIgD and to directly activate B lymphocytes can contribute to its ability to induce an IgG antibody response in vivo. Studies were performed with sets of anti-IgD antibodies that differed in their abilities to cross-link mIgD and activate B cells, but were matched for isotypic, allotypic and species determinants to eliminate foreignness for T cells as a factor that could influence the generation of an antibody response. Our observations suggest that these activities can contribute considerably to the generation of an antibody response. Comparison of three rat mAb of the same isotype and avidity for IgD indicated that two which effectively cross-link mIgD and directly activate B cells could stimulate a polyclonal IgG1 response, whereas one mAb that fails to effectively cross-link mIgD or directly activate B cells had little ability to induce polyclonal IgG1 production. Although the stimulatory and non-stimulatory rat mAb also differ in the site of the IgD molecule to which they bind, this is probably not an important determinant of their ability to induce a polyclonal IgG1 response, because three allo-anti-δ mAb, which bind to the same part of the IgD molecule as the non-stimulatory rat mAb but have greater ability to directly activate B cells, were effective inducers of polyclonal IgG1 production. Furthermore, experiments with an intact IgG2b anti-δ mAb that effectively cross-links mIgD and directly activates B cells, and its Fab/Fc fragment, which has some ability to directly activate B cells in vivo in the absence, but not in the presence of an anti-FcγRII mAb (49), also demonstrate a strong correlation between the ability to directly activate B cells and the ability to induce a T-dependent IgG1 response. These experiments clearly eliminate the possibility that the site of the IgD molecule bound is an important determinant of the ability to induce a polyclonal IgG response. Differences between the avidity of IgD binding by the intact mAb and its Fab/Fc fragment are also not likely to be important determinants of the ability to induce polyclonal IgG1 production, because 1) this would not explain why anti-FcγRII inhibits the ability of the Fab/Fc fragment to stimulate IgG1 production whereas it enhances the ability of the intact antibody to stimulate IgG1 production; and 2) IgG2a allo-anti-δ mAb that bind mIgD much less avidly than the Hδ^a/1 Fab/Fc fragment, but have some ability to directly activate B cells, are effective inducers of polyclonal IgG1 production. The importance of T cell recognition of foreignness, as well as mIg cross-linking, for anti-δ antibody induction of a polyclonal Ig response was demonstrated by the results of an experiment in which neither a rat IgG2a anti-δ mAb, which was seen as foreign by (*a* × *b* allotype)F1 mice but fails to effectively cross-link mIgD, nor an antibody to IgD of the *a* allotype, that is seen as self by these mice but effectively cross-links mIgD on their *a* allotype-expressing B cells, could stimulate a polyclonal IgG1 response in these

mice, whereas a combination of these anti-$\delta$ mAb stimulated a significant response.

Although our studies indicate that cross-linking of mIgD that results directly in B cell activation can contribute to the generation of an antibody response, they do not identify the mechanism by which this occurs. At least three different mechanisms, which are not mutually exclusive, are possible. First, the activation of the B cell by the cross-linking of its mIgD could contribute directly to its proliferation and differentiation into an Ig-secreting cell. Although mIg cross-linking alone is unable to induce Ig secretion, it can act synergistically with cytokine help to achieve this result (7, 8) and has been shown to induce increased expression of B cell receptors for T cell-produced helper factors (9). Second, the cross-linking of mIgD might enhance internalization and processing of Ag, so that more Ag could be presented in an immunogenic form to Ag-specific T lymphocytes. In vitro studies have, in fact, demonstrated that although univalent Fab fragments of anti-Ig antibodies can be internalized by the B cell, they are processed differently from intact divalent anti-Ig antibodies that have entered the cell via an mIg cross-linking mechanism in that only the divalent antibodies enter an intracellular compartment that is acidified (35, 62). Third, it is possible that changes in the activation state of the B cell that result from the cross-linking of its mIg, such as increased expression of class II MHC Ag (5), enhance its ability to present Ag to T lymphocytes even if antigen digestion per se is not affected.

Our observations appear to conflict with the results of some in vitro studies. Parker and coworkers (27, 40) have demonstrated, for example, that univalent Fab fragments of rabbit anti-mouse Ig antibody are as effective as divalent $F(ab')_2$ fragments of this antibody at mediating an interaction between mouse B cells and rabbit IgG Fab-specific helper T cells that stimulates polyclonal B cell activation and antibody production. Casten and coworkers (38, 39) have similarly found that Ag linked to Fab fragments of anti-Ig antibody is as effectively presented by B cells to T cells as is Ag linked to $F(ab')_2$ fragments of the same antibody. The apparent discrepency between these observations and ours may reflect the fact that the T cells used in the in vitro experiments were already partially activated and had been selected for high avidity for Ag, although in our in vivo system, Ag must be presented to resting T cells that do not necessarily recognize it with high avidity. It is possible that there are more stringent Ag processing and presentation requirements for the initial Ag-specific activation of T cells than for reactivation or further activation of previously activated or partially activated T cells.

*Acknowledgments.* We thank Dr. Michael Potter for his gift of mice, Drs. Ellen Vitetta, Daniel Conrad, and John Kearney for their gifts of antibodies. Dr. James Mond for his gift of reagents and helpful criticisms, and Mary Chase for her expert editorial assistance.

REFERENCES

1. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes in vitro. I. Stimulation of blast transformation with an anti-allotype serum. *J. Exp. Med. 122:423.*
2. Parker, D. C. 1975. Stimulation of mouse lymphocytes by insoluble anti-mouse immunoglobulin. *Nature 258:361.*
3. Weiner, H. L., J. W. Moorhead, and H. N. Claman. 1976. Anti-immunoglobulin stimulation of murine lymphocytes. I. Age dependency of the proliferative response. *J. Immunol. 116:1656.*
4. Sieckmann, D. G., T. Asofsky, D. E. Mosier, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med. 147:814.*
5. Mond, J. J., E. Sehgal, J. Kung, and F. D. Finkelman. 1981. Increased expression of I-region-associated antigen (Ia) on B cells after cross-linking of surface immunoglobulin. *J. Immunol. 127:881.*
6. Finkelman, F. D., I. Scher, J. J. Mond, J. T. Kung, and E. S. Metcalf. 1982. Polyclonal activation of the murine immune system by an antibody to IgD. I. Increase in cell size and DNA synthesis. *J. Immunol. 129:629.*
7. Kishimoto, T., T. Miyake, Y. Nishizawa, T. Watanage, and Y. Yamamura. 1975. Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. *J. Immunol. 115:1179.*
8. Parker, D. C., J. J. Fothergill, and D. C. Wadsworth. 1979. B lymphocyte activation by insoluble anti-immunoglobulin: induction of immunoglobulin by a T cell-dependent soluble factor. *J. Immunol. 123:931.*
9. Yaffe, L. J., and F. D. Finkelman. 1983. Induction of a B-lymphocyte receptor for a T cell-replacing factor by the cross-linking of surface IgD. *Proc. Natl. Acad. Sci. USA 80:293.*
10. Howard, M., and W. E. Paul. 1983. Regulation of B-cell growth and differentiation by soluble factors. *Annu. Rev. Immunol. 1:307.*
11. Monroe, J. G., and J. C. Cambier. 1983. B cell activation. III. B cell plasma membrane depolarization and hyper-Ia expression induced by receptor immunoglobulin cross-linking are coupled. *J. Exp. Med. 156:1589.*
12. Coggeshall, K. M., and J. C. Cambier. 1984. B cell activation. VIII. Membrane immunoglobulins transduce signals via activation of phosphatidylinositol hydrolysis. *J. Immunol. 133:3382.*
13. Pozzan, T. P., Gerslan, R. Y. Tsien, and T. J. Rink. Anti-immunoglobulin, cytoplasmic free calcium and capping in B lymphocytes. *J. Cell Biol. 94:335.*
14. Nishizuka, Y. 1986. The role of protein kinase C in cell surface transduction and tumour promotion. *Nature 308:693.*
15. Snow, E. C., J. D. Fetherston, and S. Zimmer. 1986. Induction of the c-myc protooncogene after antigen binding to hapten-specific B cells. *J. Exp. Med. 164:944.*
16. Horabeck, P., and W. E. Paul. 1986. Anti-immunoglobulin and phorbol ester induce phosphorylation of proteins associated with the plasma membrane and cytoskeleton in murine B lymphocytes. *J. Biol. Chem. 261:14817.*
17. Klaus, G. G. B., A. O'Garra, M. K. Bijsterbosch, and M. Holman. 1986. Induction of DNA synthesis in B cells by a combination of $Ca^{2+}$ ionophores and phorbol myristic acetate. *Eur. J. Immunol. 16:92.*
18. Brunswick, M., F. D. Finkelman, P. F. Highet, J. K. Inman, H. M. Dintzis, and J. J. Mond. 1988. Picogram quantities of anti-Ig antibodies coupled to dextran induce B cell proliferation. *J. Immunol. 140:3364.*
19. Wilson, H. A., D. Greenblatt, M. Pocnic, F. D. Finkelman, and R. Y. Tsien. 1987. Crosslinkage of B lymphocyte surface immunoglobulin by anti-Ig or antigen induces prolonged oscillation of intracellular ionized calcium. *J. Exp. Med. 166:601.*
20. Mond, J. J., N. Feuerstein, F. D. Finkelman, F. Huang, K. P. Huang, and G. Dennis. 1987. B-lymphocyte activation mediated by anti-immunoglobulin antibody in the absence of protein kinase C. *Proc. Natl. Acad. Sci. USA 84:8586.*
21. Dintzis, R. Z., M. H. Middleton, and H. M. Dintzis. 1983. Studies on the immunogenicity and tolerogenicity of T independent antigens. *J. Immunol. 131:2196.*
22. Mond, J. J., K. E. Stein, B. Subbarao, and W. E. Paul. 1979. Analysis of B cell activation requirements with TNP-conjugated polyacrylamide beads. *J. Immunol. 123:239.*
23. Sharon, R., P. R. B. McMaster, A. M. Kask, J. D. Owens, and W. E. Paul. 1975. DNP-lys-Ficoll: a T-independent antigen which elicits both IgM and IgG anti-DNP antibody secreting cells. *J. Immunol. 114:1585.*
24. Baker, P. J., C. E. Taylor, M. B. Faunticroy, B. W. Stashak, and Prescott. 1985. The role of antigen in the activation of regulatory cells by immune B cells. *Cell. Immunol. 96:376.*
25. Zubler, R. H., and A. L. Glasebrook. 1982. Requirement for three signals in T-independent (lipopolysaccharide-induced) as well as T-dependent B cell responses. *J. Exp. Med. 155:666.*
26. Julius, M. H., H. Von Boehmer, and C. Sidman. 1982. Different tion of two signals required for activation of resting B cells. *Proc. Natl. Acad. Sci. USA 79:1989.*
27. Tony, H. P., N. E. Phillips, and D. C. Parker. 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated, major histocompatibility-restricted T cell-B cell cooperation. *J. Exp. Med. 162:1695.*
28. Chesnut, R., and H. M. Grey. 1981. Studies on the capacity of B cells to serve as antigen-presenting cells. *J. Immunol. 126:1075.*
29. Chesnut, R., S. Colon, and H. M. Grey. 1982. Requirement for processing of antigens by antigen presenting B cells. *J. Immunol. 129:2382.*

30. Kakiuchi, T., R. W. Chesnut, and H. M. Grey. 1983. B cells as antigen presenting cells: the requirement for B cell activation. *J. Immunol. 131:109.*
31. Tony, H. P., and D. C. Parker. 1985. Major histocompatibility complex-restricted, polyclonal B cell responses resulting from helper T cell recognition of anti-immunoglobulin presented by small B lymphocytes. *J. Exp. Med. 161:223.*
32. Rock, K. L., B. Benacerraf, and A. K. Abbas. 1984. Antigen presentation by antigen-specific B lymphocytes. *J. Exp. Med. 160:1102.*
33. Abbas, A. K., S. Haber, and K. L. Rock. 1985. Antigen presentation by hapten-specific B lymphocytes. II. Specificity and properties of antigen presenting B lymphocytes and function of immunoglobulin receptors. *J. Immunol. 135:1661.*
34. Lanzavecchia, A. 1985. Antigen-specific interactions between T and B cells. *Nature 314:537.*
35. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted lymphocytes. *Annu. Rev. Immunol. 8:773.*
36. Prohman, M., and C. Cowing. 1985. Presentation of antigen by B cells: functional dependence on radiation dose, interleukins, cellular activation, and differential glycosylation. *J. Immunol. 134:2269.*
37. Krieger, J. I., S. F. Grammer, H. M. Grey, and R. W. Chesnut. 1985. Antigen presentation by splenic B cells: resting B cells are ineffective, whereas activated B cells are effective accessory cells for T cell responses. *J. Immunol. 135:2937.*
38. Casten, L. A., E. K. Lakey, M. L. Lelachich, E. Margoliash, and S. K. Pierce. 1985. Anti-immunoglobulin augments the B-cell antigen-presentation function independently of internalization of receptor-antigen complex. *Proc. Natl. Acad. Sci. USA 82:5890.*
39. Casten, L. A., P. Kaumaya, and S. K. Pierce. 1988. Enhanced T cell responses to antigenic peptides targeted to B cell surface Ig, Ia, or Class I molecules. *J. Exp. Med. 168:171.*
40. Gosselin, E. J., H.-P. Topy, and D. C. Parker. 1988. Characterization of antigen processing and presentation by resting B lymphocytes. *J. Immunol. 140:1408.*
41. Warren, H. S., F. R. Vogel, and L. A. Chedid. 1986. Current status of immunological adjuvants. *Annu. Rev. Immunol. 4:369.*
42. Finkelman, F. D., I. Scher, J. J. Mond, S. Kessler, J. T. Kung, and E. S. Metcalf. 1982. Polyclonal activation of the murine immune system by an antibody to IgD. II. Generation of polyclonal antibody production and cells with surface IgD. *J. Immunol. 129:638.*
43. Weber, R. J., and F. D. Finkelman. 1987. Increased expression of the B lymphocyte receptor for transferrin is stimulated by in vivo crosslinking of cell surface IgD. *Cell. Immunol. 104:400.*
44. Finkelman, F. D., J. Ohara, D. K. Goroff, J. Smith, N. Villacreses, J. J. Mond, and W. E. Paul. 1986. Production of BSF-1 during an in vivo, T-dependent immune response. *J. Immunol. 137:2878.*
45. Finkelman, F. D., T. R. Malek, E. M. Shevach, and J. J. Mond. 1986. In vivo and in vitro expression of an interleukin 2 receptor by murine B and T lymphocytes. *J. Immunol. 137:2252.*
46. Goroff, D. K., A. Stall, J. J. Mond, and F. D. Finkelman. 1986. In vitro and in vivo B lymphocyte activating properties of monoclonal anti-δ antibodies. I. Determinants of B lymphocyte activating properties. *J. Immunol. 135:2381.*
47. Zitron, I. M., and B. L. Clevinger. 1980. Regulation of murine B cells through surface immunoglobulin. I. Monoclonal anti-δ antibody that induces allotype-specific proliferation. *J. Exp. Med. 152:1135.*
48. Parham, P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. *J. Immunol. 131:2895.*
49. Goroff, D. K., and F. D. Finkelman. 1988. Activation of B cells in vivo by a Fab/Fc fragment of a monoclonal anti-IgD antibody requires an interaction between the antibody fragment and a cellular IgG Fc receptor. *J. Immunol. 140:2919.*
50. Unkeless, J. C. 1979. Characterization of a monoclonal antibody against mouse macrophage and lymphocyte Fc receptors. *J. Exp. Med. 153:1198.*
51. Stall, A. M., and M. R. Loken. 1984. Allotypic specificities of murine IgD and IgM recognized by monoclonal antibodies. *J. Immunol. 132:787.*
52. Bazin, H., Ed. 1990. *Rat Hybridomas and Rat Monoclonal Antibodies.* CRC Press, Boca Raton, FL.
53. Rao, M., W. T. Lee, and D. H. Conrad. 1987. Characterization of a monoclonal antibody directed against the murine B lymphocyte receptor for IgE. *J. Immunol. 138:1845.*
54. Kappler, J. W., B. Skidmore, J. White, and P. Marrack. 1981. Antigen-inducible, H-2 restricted, interleukin-2-producing T cell hybridomas: lack of independent antigen and H-2 recognition. *J. Exp. Med. 153:1198.*
55. Finkelman, F. D., J. Holmes, J. F. Urban, W. E. Paul, and I. M. Katona. 1989. T help requirements for the generation of an in vivo IgE response: a late acting form of T cell help other than interleukin 4 is required for IgE but not for IgG1 production. *J. Immunol. 142:403.*
56. Mountz, J. D., J. F. Mushinski, J. D. Owens, and F. D. Finkelman. 1990. The in vivo generation of murine IgD-secreting cells is accompanied by deletion of the $C_\mu$ gene and occasional deletion of the gene for the $C\delta_1$ domain. *J. Immunol. 145:1583.*
57. Sidman, C. L., and E. R. Unanue. 1976. Control of B lymphocyte function. I. Inactivation of mitogenesis by interactions with surface immunoglobulin and Fc receptor molecules. *J. Exp. Med. 144:882.*
58. Scribner, D. J., H. L. Weiner, and J. W. Moorhead. 1978. Anti-immunoglobulin stimulation of murine lymphocytes. V. Age related decline in Fc receptor-mediated immunoregulation. *J. Immunol. 121:377.*
59. Herzenberg, L. A., S. Black, T. Tokuhishi, and L. A. Herzenberg. 1980. Memory B cells at successive stages of differentiation: affinity maturation and the role of IgD receptors. *J. Exp. Med. 151:1071.*
60. May, R. D., F. D. Finkelman, H. T. Wheeler, J. W. Uhr, and E. S. Vitetta. 1990. Evaluation of ricin A chain-containing immunotoxins directed against different epitopes on the δ-chain of cell surface-associated IgD on murine B cells. *J. Immunol. 144:3637.*
61. Unkeless, J. C., H. Fleit, and I. S. Mellman. 1981. Structural aspects and heterogeneity of immunoglobulin Fc receptors. *Adv. Immunol. 31:247.*
62. Metezeau, P., I. Elguindi, and M. E. Goldberg. 1984. Endocytosis of the membrane immunoglobulin of the mouse spleen B cells: a quantitative study of its rate, amount and sensitivity to physiological, physical and crosslinking agents. *EMBO J. 3:2235.*